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Solid Phase Synthesis of Modular Peptide-based Targeted Molecular Imaging Agents

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Solid Phase Synthesis of Modular Peptide-based

Targeted Molecular Imaging Agents

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CERTIFICATE OF APPROVAL

M.S. DEGREE THESIS

The M.S. Degree Thesis of Xinyu Xu has
been examined and approved by the thesis
committee as satisfactory for the thesis required for
the M.S. degree in Chemistry.

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Date

Abstract

Targeted molecular imaging agents (TMIA) are emerging as useful tools for early diagnosis of cancer and other diseases. These agents couple imaging agents such as near infrared fluorescence (NIRF) dyes or metallic contrast agents such as gadolinium (Gd) used in magnetic resonance imaging (MRI) to targeting agents that bind to biomarker receptors in cancer cells. Our group has developed a modular synthesis of peptide-based TMIAs containing these two agents starting from "puzzle pieces". Puzzle pieces, or modules, are amino acids with imaging groups bonded to their side chains. These are assembled together to form imaging peptides which are then conjugated to targeting groups. The research goal was to synthesize targeting peptides using solid phase peptide synthesis (SPPS), and then add the imaging puzzle pieces to these in the same SPPS method. SPPS is widely used and has many advantages in the synthesis of TMIAs. The first goal, to learn how to synthesize simple peptides by SPPS, was accomplished. The second goal of making Met-enkephalin, a bioactive penta-peptide, and conjugating the imaging puzzle pieces containing a NIRF dye or gadolinium chelate for MRI by SPPS was also successful. The final goal, to synthesize a deca-peptide, 18-4a, useful for targeting breast cancer and then to couple these same imaging puzzle pieces, to the peptide 18-4 in the last step, was also accomplished.

Acknowledgements

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I would like to show my gratitude to Dr. Kamaljit Kaur from Chapman University for providing our first sample of the breast cancer peptide 18-4a, and for her help in mentoring me and my adviser in the choice of which peptide to synthesize and help with the actual synthetic design. I would also like to show my gratitude also to Dr. Justin Miller of Hobart College for his help in the techniques of SPPS including a manual for synthesis prepared in his lab that he shared with us.

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Lastly, my family has been very supportive in my graduate studies and I would like to sincerely thank them from the bottom of my heart.

Abbreviations

AA (Amino Acids)
ACN (Acetonitrile, an organic solvent used in HPLC)
Boc (Tert-butyloxycarbonyl, a protecting group removed in strong acid)
CFM (Confocal fluorescence microscopy)
DCM (Dichloromethane)
DEA (Diethylamine, a de-protecting agent for Fmoc)
DIPA (Diisopropyl ethyl amine, also called DIEA, used as a base in coupling reactions)
DMF (N,N-dimethylformamide, a solvent for peptide coupling)
DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid)
Fmoc (Fluorenylmethyloxycarbonyl, a common peptide protecting group)
HATU (A common peptide coupling reagent)
HPLC (High Pressure Liquid Chromatography)
LC-MS (liquid chromatography – mass spectrometry)
MeOH (Methanol an organic solvent used in HPLC)
MRI (Magnetic Resonance Imaging)
MS (Mass Spectrometry)
Mtt (Methyltrityl, a protecting group that is easily removed with mild acid)
NIR (Near-Infrared)
NIRF (Near-Infrared Fluorescent)
NIRQ (Near-Infrared Quencher)
NMM (N-methylmorpholine, used as a base in coupling reactions)
NMP (N-methylpyrolidine, a peptide coupling solvent)
PAI (Photoacoustic Imaging)
PCa (Prostate Cancer)
PET (Positron Emission Tomography)
λ_{max} (Lamda Max, peak wavelength in UV-Vis-NIR absorption spectroscopy
TBTU (A common peptide coupling reagent)
TCA Targeted contrast agent
TFA (Trifluoroacetic acid, a common buffer, also used to remove protecting groups)
THF (Tetrahydrofuran, an organic solvent)
TMIA (Targeted molecular imaging agent)
TSTU (A common peptide coupling reagent)
UV-Vis-NIR (Ultra Violet – Visible – NIR absorption spectroscopy)

Amino Acids Abbreviations

Amino Acids	Three Letter Abbreviations	One Letter Abbreviations
Alanine	Ala	A
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Tryptophan	Try	W
Tyrosine	Tyr	Y
Norleucine	Nle	X

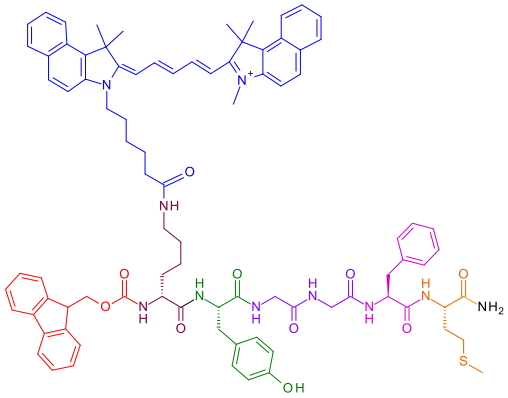
Information on NIR dyes utilized

Cy5.5: NIRF dye absorbing at 680 nM and fluorescing at 710 nM, useful in confocal fluorescence microscopy (CFM).

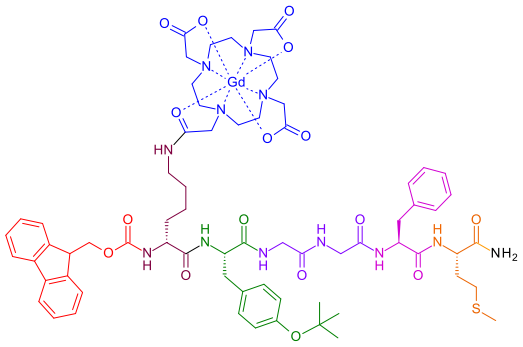
Nomenclature of Peptides containing Dyes and Metals

In peptide synthesis, the structures are synthesized from right to left, but named in the left to right by convention. In solution phase synthesis, the C-terminal carboxylic acid on the right, may be changed to a C-terminal amide, to make it non-reactive. The nitrogen on each amino acid coupled is most often protected by an Fmoc group. This nitrogen and protecting group on the left side of the peptide chain is referred to as the N-terminus.

In peptide nomenclature, the three letters abbreviation is the standard utilization for amino acids. Side chains are drawn alternately up and down. Natural amino acid is a solid wedge when up and dashed when down. Unnatural is the opposite. Unnatural amino acids are prefaced by d in three letter abbreviations, and are lower-case letters in one-letter abbreviations. Unnatural amino acids are put in to give proteolytic stability. A protecting group should be brought in on the amines of side chains. These protecting groups are written in parentheses. We introduced a convention of putting the imaging groups, likewise, in parentheses. This is important since our imaging groups are added on the side chains of the amino acids. The illustrations represent the peptide, Met-enkephalin, with the dye Cy5.5 or with the metal-chelate Gd-DOTA on the side chain of d-Lysine.



Fmoc-dLys(Cy5.5)-Tyr- Gly-Gly-Phe-Met-NH_2



Fmoc-dLys(Gd-DOTA)-Tyr(tBu)-Gly-Gly-Phe-Met-NH_2

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Chapter 1. Background Information

1.a. Cancer and Early Detection

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body.[1, 2] Our lab is focused on discovering new ways to image cancer, specifically prostate cancer (PCa) and breast cancer (BrCa). Cancer starts when cells in the these or other organs begin to grow out of control. These cells usually form a mass, or tumor that can often be seen with various imaging modalities such as X-ray in a mammogram, or they can be felt as a lump upon examination.

There were approximately 18.1 million new cases and 9.6 million deaths in 2018. One in 5 men and one in 6 women worldwide develop cancer during their lifetime, and one in 8 men and one in 11 women will die from cancer.[3]

One of the main goals of this research project was to develop a method for the synthesis of targeted molecular imaging agents (TMIAs) for breast cancer. For females, breast cancer is the second main cause of death, just after lung cancer.[4] In 2017, 252,710 new diagnoses of breast cancer occurred in females, and 40,610 females died from these diseases.[5] Since breast cancer is associated with many factors, such as age, genetics, a family history of breast cancer or breast lumps, alcohol consumption, radiation exposure, hormone treatment and dense breast tissue, it is important to be aware of the symptoms and how to recognize them, and it is important to undergo regular screening (i.e. mammograms).[5] The best hope is to detect the cancer early, in order to improve the outcome and to reduce the risk of death.

Molecular imaging is a multidisciplinary science that is developed rapidly recently which involves in chemistry, biology and medicine.[6] As a result of advances in molecular

imaging, more and more researches has focused on the need for a variety of safe and effective targeting molecular imaging agents (TMIAs). The key purpose is to visualize disease in real time and noninvasively assess physiological or pathological processes in the living organisms. Molecular imaging is a powerful tool that can detect the disease in the early onset which could save more lives. But since it is looking for small changes, it requires highly sensitive instruments and techniques to detect the specific molecular processes in diseased cells and tissues.

Confocal fluorescence microscopy (CFM) is a microscopy technique that provides three-dimensional optical resolution, which is coupled with a laser light source, diffraction-limited optics and a high-efficiency detection system has been used to study the diffusive movement and emission process of individual fluorescent molecules in the liquid phase at room temperature.[7] Because we have a relatively newly acquired CFM instrument in the College of Science, and have set up a collaboration with Dr. Evans, our primary focus in imaging has been to synthesize TMIAs which make use of a fluorescent dye. The dye we chose is Cy5.5 is chosen because it falls perfectly in the range of the CFM instrument with excitation at 633 nm, which is the wavelength of the excitation laser, and the range of emission that is entirely captured between 650 nm and 750 nm. In our approaches Cy 5.5 is conjugated to targeting systems for prostate and breasts cancer followed by evaluation by CFM.

In addition to CFM the other two modalities of interest in our lab are magnetic resonance imaging (MRI) and to synthesizing proto-types for positron emission tomography (PET). Targeted contrast agents (TCAs) for NMR make use of chelated gadolinium metal and TMIAs for PET make use of radioactive metals including gallium

(Gd) , indium (In), yttrium (Y) and copper (Cu). In our facilities radioactive agents are not allowed so this will not be a focus of my research. However, the results we obtain for Gd for MRI could be applied to the synthesis of PET agents containing other chelated metals that will be useful to others in related future research.

1.b. Modular "Puzzle Piece Approach" (peptides)

Targeted molecular imaging is emerging as a useful method for early diagnosis of cancer and other diseases.[8] These agents couple various imaging agents such as near infrared fluorescence (NIRF) dyes or metallic contrast agents used in magnetic resonance imaging (MRI) with targeting agents. The targeting agents are designed to bind to unique biomarker receptors in cancer cells, and therefore highly selective at "lighting up" both cancer cells.

Our group has developed a modular synthesis of peptide-based TMIAs starting from "puzzle pieces".[9,10] Puzzle pieces are amino acids with imaging groups bonded to their side chains. These are assembled together to form imaging peptides which are then conjugated to targeting groups to prepare TMIAs as shown in Figure 1 on the left side. So far these have been synthesized by solution phase peptide synthesis. In this method the yields are low, and synthesis requires many steps including purification of each intermediate.

The goal of my research is to synthesize targeting peptides by a technique that is new to our group at RIT which is solid phase peptide synthesis (SPPS), and to incorporate the imaging modules or "puzzle pieces" as part of the synthesis to assemble TMIAs. SPPS is widely used and has many advantages as outlined below. The difference in producing TMIAs from a SPPS versus our group's method is that in the SPPS method, the targeting

peptide is prepared first, followed by incorporation of the imaging modules as shown on the right side of Figure 1. In the established group method, the imaging modules are prepared first, followed by conjugating the targeting group on last.

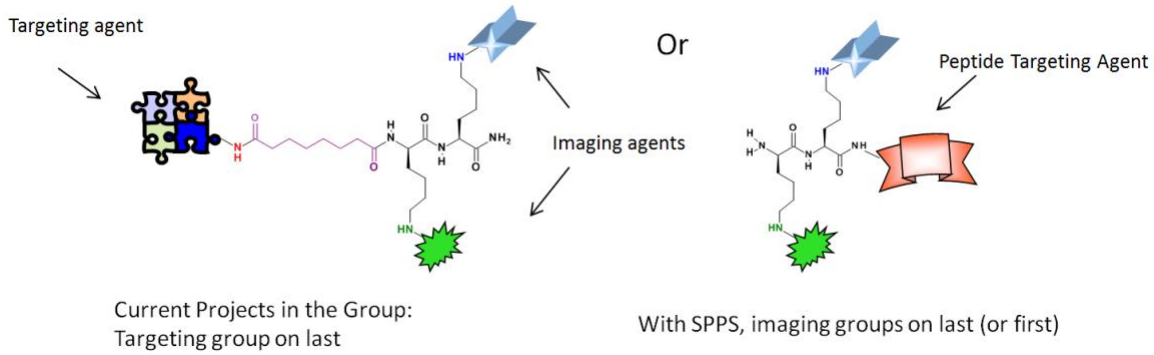


Figure 1: Synthesis of TMIAs using solution phase synthesis (left) and SPPS (right)

There are distinct benefits from using low-molecular weight (<3,000) peptides for both targeting and imaging agents. Small peptides have well-known bioavailability, with known advantages in pharmacokinetics, tissue penetration, metabolism and clearance that provide beneficial aspects in efficacy and safety.[11] A disadvantage is that peptides can be cleaved by proteases not only in the digestive tract, but also by proteases in the blood in the case of injectable TMIAs such as those in this project. However, the proteolytic stability can be increased by incorporation of D amino acids at critical centers to optimize circulation time and clearance. Rapid metabolism and clearance is a desirable factor when using injectable imaging agents, so D amino acids can further be used to fine-tune the stability in blood to last a required amount of time to match the imaging method. For example an MRI study might take about 30 minutes.

The goal of producing TMIAs with SPPS was pursued by a plan that included synthesizing small (mono- to tri-peptides), then medium peptides (such as penta-peptides)

that would emulate targeting peptides, then to couple each of these to the dye and metal imaging modules in the last step. The stretch goal was to synthesize an actual targeting deca-peptide for breast cancer and likewise couple these to the dye and metal modules containing Cy5.5 and Gd(III).

Carrying out this plan, the first goal was to learn how to synthesize simple mono-, di- and tripeptides from common amino acids. The majority of synthesis time in this project was devoted this goal. It involved troubleshooting, optimizing and essentially teaching ourselves how to synthesis peptides utilizing SPPS, then bonding the imaging modules or puzzle pieces to the peptides while still on the SPPS resin or after cleavage from the resin.

The second goal was to apply this optimized method to the synthesis of a small, bio-active peptide. We chose Met-enkephalin, a natural opiate receptor targeting peptide, and likewise assembled these with the Cy5.5 and Gd-DOTA imaging modules.

The third goal (a stretch goal) was to synthesize breast cancer targeting peptides of the type discovered by Dr. Kaur at Chapman University[12–14] and attach the same imaging modules ("puzzle pieces") in the last step. All of the steps including the imaging module couple would be accomplished by SPPS. Progress toward these goals will be described in the next section.

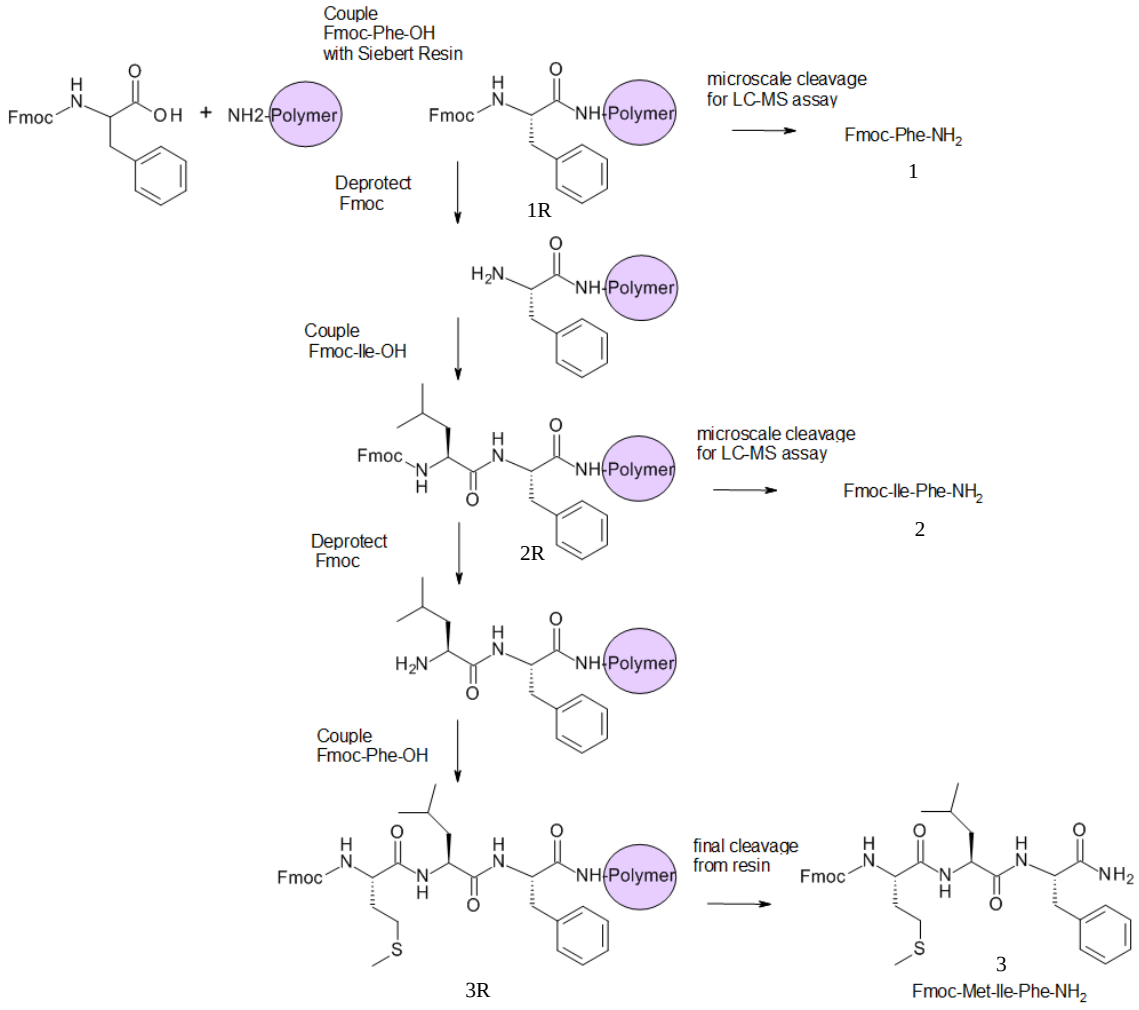
1.c. Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) is a method in which molecules are bound to a bead and synthesized step-by-step using solutions of reagents that act on these bound peptides.[15] Compared with synthesis in a liquid state, also called solution phase peptide synthesis, it is easy to remove excess reactant or byproduct by simply washing the beads.

The advantage of solid phase peptide synthesis is that the products can be isolated easily since all the intermediate peptides are immobilized on a polymer such as polystyrene. Thus, the products can be purified by filtration and washing. Repetition of the deprotection-coupling process will lead to larger peptides.[16] In general, SPPS should give good yield and high purity. It is a great way to synthesize long peptide chains.

A typical synthesis using SPPS is shown in Scheme 1 (below). In SPPS, the first step is to bind the first amino acid (synthesizing from right to left) to the polymeric resin by a coupling reaction of the free acid on the amino acid and the amine on the polymeric resin. This is followed by deprotection of an Fmoc group (shown later). Fmoc is used routinely in SPPS as a primary protecting group for the backbone amine. The second step after coupling each amino acid to the resin is to remove the Fmoc group by the use of a secondary amine, typically piperdine to yield the free N-terminus amine which is then ready to couple to the next amino acid.

By repeating the process of bringing in an Fmoc protected amino acid, coupling it to the prior deprotected amine bound to the resin, and deprotecting the Fmoc again, a longer peptide chain can be synthesized as shown in Scheme 1. As described in the results section, tripeptide Fmoc-Phe-Ile-Phe-NH_2 was prepared as part of the SPPS learning and development phase of the project.



Scheme 1: Solid Phase Peptide Synthesis of a Tripeptide

For following the progress of reactions, the classic Kaiser test is a qualitative method that simply shows if the coupling reaction is completed by detecting unreacted amines after micro-cleavage of a small portion of the beads. However, because we had access to liquid chromatography-mass spectroscopy (LC-MS) right in the lab, we found it most effective to conduct a micro-cleavage reaction (described later) and directly assay each product. This, and other details of the SPPS methods that we developed are described in detail in the following section.

Chapter 2. Solid Phase Peptide Synthesis (SPPS) Methods

2.a. Choice of SPPS resin

The first step to build up a peptide chain is to find the best linker resin. Historically the Merrifield resin was invented as the first resin. Using this resin, the final cleavage produces the C-terminal acid of the peptide. The resin most currently used is a modification of this resin called the Wang resin. If a C-terminal amide is required, the resin most commonly used is currently the Rink-amide resin.

All of these resins require pure TFA to cleave the peptide product. But because our near infrared dyes (NIR dyes) used in medical imaging are decomposed by strong acid, which means it needs to find milder conditions. Sieber resin, also known as xanthenyl linker resin, is the only resin commercially available at an affordable price that permits dilute acid (1-2 % TFA) for cleavage. The structure of the Xanthenyl containing the linker moiety, where the amine is protected by an Fmoc group, is shown in Figure 2.

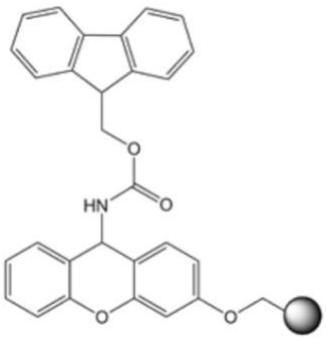


Figure 2: The structure of Sieber resin (Xanthenyl linker resin)

2.b. Calculation of micro-equivalents of amine on resin

In order to use the correct stoichiometry for each reaction on a SPPS resin, it is important to determine the micro-equivalents of reactive amine on the resin, upon which

the peptide is built. This is accomplished by deprotecting the Fmoc group from the entire batch of resin to be coupled, which in our case is generally 100 mg per synthesis, followed by measuring the absorbance of the resultant Fmoc byproduct, which as a known extinction coefficient ($M^{-1}cm^{-1}$) at a key absorption band of 300 nm.

After an extensive series of trials in which many conditions were explored, the following set of conditions was found to provide the best results.

First, the xanthenyl resin, which was chosen as the most appropriate resin which would be compatible with the acid-sensitive dyes we ultimately used, was pre-swelled for about twenty minutes before the experiment starts. Pre-swelling can help resin improve accessibility within their macroporous structure. DMF is used as the organic solvent to pre-swell it.

Conditions for removal of the FMOC (Fluorenylmethoxycarbonyl) group from the resin were optimized by a reported method which used 2% DBU, 5% saturated piperazine, and 93% DMF in order to completely deprotect it from the resin.[17] To ensure the FMOC has been removed completely, about 3mL of deprotection solution was added to the resin and stirred for 5 minutes, then repeated twice. All deprotection solutions were drained, combined and diluted as described below to test the concentration of the resin. This optimized Fmoc deprotection process was tested by the UV absorption method several times and was found to be the most efficient way to remove all Fmoc groups.

The combined deprotection solutions should be diluted in ethanol, or the absorbance will be too high to measure. 30uL of deprotection solution was diluted in 2.97mL of ethanol, mixed well and assayed by UV spectroscopy to measure the absorbance at 300nm and calculate the equivalents of the solution. The concentration equals

absorbance divided the extinction coefficient and the light path length as calculated by the equation below (Beer's Law).

$$Abs = \varepsilon lc \rightarrow c = \frac{Abs}{\varepsilon l}$$

2.c. Preparation of deprotected resin for synthesis

After determination of the micro equivalents per 100 mg, which is our initial "reaction scale", the SPPS resin needs to be further prepared in order to be utilized in the next reactions. So, the beads are next washed with DMF to remove all the deprotection solution by applying 2mL DMF and stirring for 30 seconds, apply air pressure to remove the eluent through the frit in the SPPS vessel, then repeating three times. Next, using the equivalents number to calculate the mass of all the compounds that are needed, the amino acid is first activated, then the solution transferred to be the beads and stirred for at least 45 minutes to make sure the coupling is complete.

The optimized stoichiometry of the coupling reaction was found to be 1.9 equivalents of coupling reagent (HATU), 2.0 equivalents of amino acid and 10 equivalents of base (DIEA). The specific order of addition is to dissolve the amino acid in DMF first, followed by DIEA, followed by adding HATU which was separately dissolve in DMF first. The mixture is transferred to the reaction vial and let it couple for 45 minutes.

After the coupling reaction, the beads were washed with 2mL of DMF, air pressure was applied to remove the eluent through the frit in the SPPS vessel, and this procedure was repeated twice to remove all the unreacted compounds, base and starting materials. This is the clear advantage of SPPS in which the growing peptide chain stays on the beads and all starting materials and impurities are easily washed off.

We developed two different procedures to cleave the final peptides. The first procedure is more applicable to simply determine if we have synthesized the correct intermediate or to learn if the targeting peptide has been made. In this procedure a small portion of the resin was removed in order to assay the product at that step. In this case we are not concerned about the yield, but to identify and assess the purity of the intermediate peptide or product.

For the cleavage process, the beads should be washed with DCM three times beforehand to remove all the DMF in the resin. By the first method, a small portion (ca. 1-2 mg) of beads are removed and stirred with 1 mL of 1% TFA in DCM for 45 minutes, then filtered and concentrated on a rotovap, then dissolved in methanol for analysis by LC-MS.

The second method is better in the case where yields needs to be determined and is generally on a larger portion of the beads or the entire amount. In this procedure, 1mL of 1% TFA in DCM is added to the resin, stirred for 45 seconds or 1 minute, then this process is repeated twenty times to make sure all the product has been cleaved. The solution was drained and concentrated on a rotovap in a pre-weighed flask for yield determination. An aliquot of this may be removed for LC-MS analysis.

It is important to note that yields are subject to considerable loss due to the micro-cleavage and LC-MS analysis at each step as in the first procedure, as well as removal of aliquots for various analyses in the final step. This is also noted below in the philosophy of yields section. In general, the focus of this research was to develop synthetic routes to peptides and TMIAs and not to focus on yields. In order to accomplish this purity needs to be examined at each step. Once a procedure has been established, it is more common to

run through the entire SPPS on a larger scale and measure the yields and purities of peptides using the optimized procedure. This will be continued by future students.

2.d. General procedure for the optimization of peptides

At the beginning of learning SPPS, we tried to synthesis a tripeptide Fmoc-Phe-Ile-Phe-NH_2 as the first goal. To deprotect the Fmoc group, the most common reagent, 20% piperidine and 80% NMP was used. After several attempts, there were always many impurities in the product, such as Fmoc-Phe-Phe-NH_2, Fmoc-Ile-Phe-NH_2.

It is important to note that these can be caused by two incomplete processes: by either incomplete Fmoc deprotection or incomplete coupling, or both. These can lead to the impurities as described in Figure 3.

Figure 3: The origin of impurities (Pol=polymer resin), red is desired product.

The first guess at the flaw in synthesis leading to the impurities was incomplete coupling of the first amino acid to the resin. This was deduced because the second coupling amino acid was found as its terminal amide form after coupling process. This indicates there was free amine on the resin even after the first coupling which would give rise to the second amino acid (monopeptide) in its amide form. In order to solve this problem, the equivalents of each reagent were changed several times.

With the help of Dr. Justin Miller from Hobart College, the number of equivalents of the coupling reagent was advised to be a little bit less than the amino acid, so this was adjusted to 2 equivalents of amino acid and 1.9 equivalents of coupling reagent (HATU, described later).

In addition to the change in equivalents of coupling agent, after several more experiments, it was determined that the use of 10 equivalents of diethylpropylamine was more favorable than 20. Although these combined changes gave better result than before, there were still impurities contained in the product.

Due to the above results, the second consideration was the incomplete Fmoc deprotection both in the initial deprotection of the resin, and the deprotection of each amino acid in the SPPS. The mechanism of this deprotection is shown in Scheme 2 (below). Because the Fmoc deprotection time was ten minutes initially, in order to make sure the process was finished completely, the deprotection time was extended to twenty minutes. Unfortunately, the result was not changed.

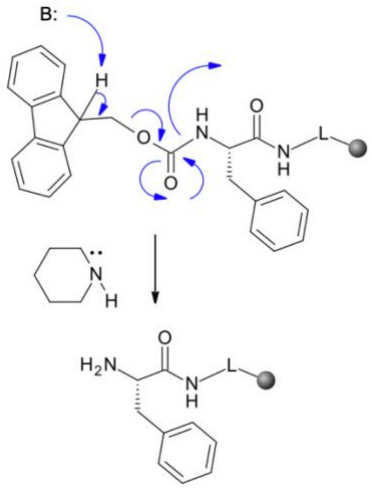
By searching the literature, an alternative protocol for the standard deprotection by piperidine or diethylamine was found and tried. The new conditions, 2% DBU, 5% Piperazine and 93% DMF mixture solution,[17] gave remarkable results and were deemed as

giving the best procedure to remove the Fmoc group. It reacted very rapidly. Two minutes were enough to remove all the Fmoc groups. But just to make sure no more Fmoc group has still linked to the previous peptide, five minutes of deprotection time was repeated twice.

After changing all of these methods, there were almost no impurities in the product anymore. The conclusion of this optimization campaign was that the incomplete Fmoc deprotection had been the main problem that caused impurities formed. With the new combination of optimized coupling and optimized deprotection of Fmoc, almost all of the subsequent peptide syntheses were successful yielding pure di-peptides, tri-peptides and later longer peptides all the way up to deca-peptides.

2.e. Mechanism of the deprotection of Fmoc

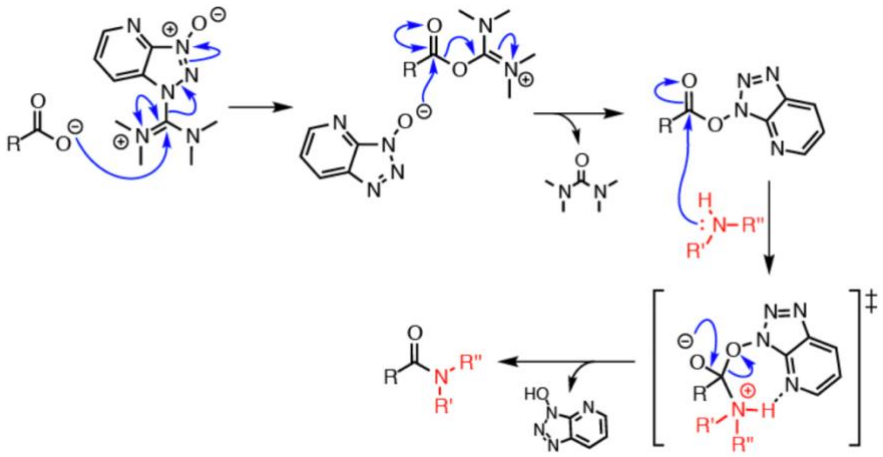
At every step in SPPS, it is most common to use the fluorenylmethyloxy carbonyl (Fmoc) group to protect the backbone N-terminal nitrogen of each in-coming amino acid. This may be effectively removed by bases such as piperidine, diethyl amine or a recipe we discovered in the literature using piperazine and DBU. The advantage is that acid sensitive groups may be used on the side chains of amino acids to be able to differentiate those groups. This is called "differential or orthogonal protection" strategy in SPPS. The mechanism of removal of the Fmoc group by the base is shown below.



Scheme 2: Mechanism of Fmoc deprotection

2.f. Mechanism of coupling of amino acids by HATU

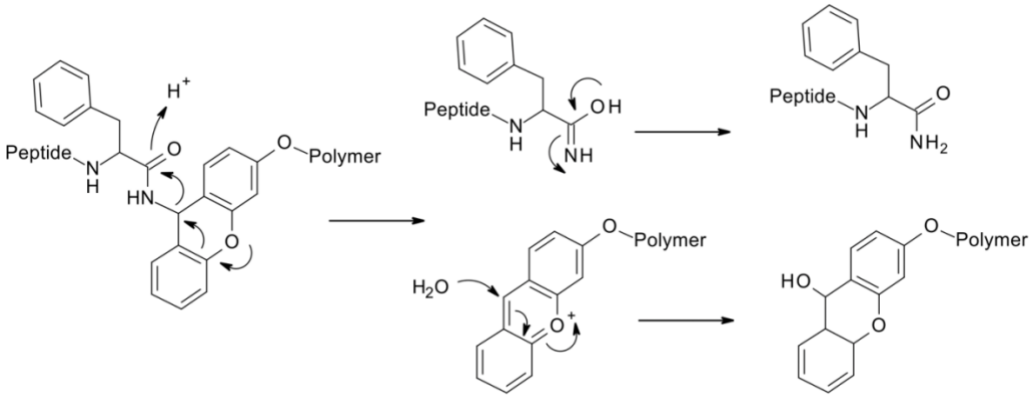
The mechanism of coupling by HATU is shown in Scheme 3. In this reaction the intermediate is an "activated ester" based on a pyridyl triazole, also known as oxy pyridyltriazole (OPT) ester.



Scheme 3: Mechanism of amino acid coupling with HATU

2.g. Mechanism of cleavage of peptide from the Xanthenyl

To remove the final peptide from the resin a cleavage process using dilute trifluoroacetic acid (TFA) was optimized. Due to the sensitivity of dyes and metal chelates in our imaging agents, it was important to use very mild acid, such as 1-2 % TFA in DCM, and this is why the Sieber resin was chosen as described earlier. The mechanism of cleavage from the xanthenyl linker in the Sieber resin is shown in Scheme 4. In this reaction the xanthenyl heterocycle is prone to decomposition followed by cleavage by the mechanism shown.



Scheme 4: Cleavage of peptide from the Sieber (Xanthenyl) Resin with 1 or 2 % trifluoroacetic acid (TFA) yields a C-terminal amide

Chapter 3. Optimized Step-by-step recipe for the SPPS of peptide

In lieu of a detailed experimental section for the generic synthesis of peptides, it was decided that a recipe, or set of directions, for the synthesis by SPPS, would be more useful for future students in the molecular imaging lab (MIL). The recipe was therefore formulated as described below. Reagent acronyms have also been defined in the abbreviations section.

3.a. Pre-Swelling

Weigh out 100 mg of Xanthenyl linker resin, transfer it to the SPPS reaction vessel. Then, add a stir bar into the vessel. Use a syringe to add 1~2mL of N,N-Dimethylformamide (DMF) into the vessel. Turn on the stir plate and let it stir for about 20 minutes. Drain the vessel by applying some air pressure to help.

3.b. Fmoc Deprotection

Mix 2% 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), 5% piperazine and 93% DMF together to make the deprotection solution, shake it well. Use a glass pipette to add 1mL of the deprotection solution into the SPPS vessel, let it stir for 5 minutes. Then use a 20mL scintillation vial to collect the deprotection solution by draining the vessel into a vial. Repeat it two more times. Save the collected deprotection solution.

3.c. Washing after Deprotection

Add about 2mL of DMF to the vessel, stir for 30 seconds and drain it. Repeat these 3 times to make sure all the deprotection solution has been removed.

3.d. Calculation of Equivalents

Prepare three 3mL quartz cuvette, place 3mL 200 proof ethanol in each cuvette. Two of them as blank. Using a syringe to remove 10μL of ethanol from the third cuvette

and place 10μL of eluate from the scintillation vial into the cuvette. Mix it well. Use the cuvette which only contains ethanol to blank the instrument. Then place the cuvette in UV spectrophotometer. Set the wavelength range from 200nm to 500nm. Record the absorbance at 300nm. Use the formula $Abs = \varepsilon lc$ and the Excel sheet to calculate the concentration of the deprotection solution. Record the result.

Example:

$$Abs = \varepsilon lc \rightarrow c = \frac{Abs}{\varepsilon l} = \frac{0.633}{7800\ M^{-1}cm^{-1} \times 1\ cm} = 0.0000811\ M$$

$$300X = 300 \times 0.0000811\ M = 24.3\ mM$$

$$24.3\ mM = 24.3\ \mu mol/mL = 73.0\ \mu mol/3mL = 73.0\ \mu mol/100mg\ of\ resin = 73.0\ \mu mol/0.1g = 0.730\ mmol/g$$

Table 1: Calculation of Equivalents

Xanthenyl				
εl (M^{-1})	Absorbance	Concentration (M)	Concentration (mM)	Equivalents (mmol/g)
7800	0.633	8.12×10^{-5}	24.3	0.730

3.e. Coupling Amino Acids

Weigh out 2.0 equivalents of the amino acid, 1.9 equivalents of the coupling reagent 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) in separate scintillation vials. Add DMF to the two vials to dissolve the amino acid and coupling reagent. Use a glass pipette to add 10 equivalents of N, N-Diisopropylethylamine (DIEA) to the vessel which contains amino acids. Then use another glass pipette to transfer all the coupling reagent solution to the vial containing

amino acid and DIPEA Shake it or stir it for one minute and transfer the mixture solution to the reaction vial. Stir for 45 minutes.

3.f. Washing after Coupling

Apply air pressure to drain the vial. Add about 2mL of DMF into the vial, stir for 30 seconds and drain it. Repeat three times.

3.g. Synthesizing Longer Peptide Chains

Repeat the process from Fmoc deprotection to washing after coupling until the targeting peptide has been made. If it is not possible to finish making the targeting peptide in one day, after the coupling washing step, add 1mL of dichloromethane (DCM) to wash the beads and drain it. Label and store it properly in the fridge for next-day use. On the next day, let the peptide warm up in room temperature, add some DMF to wash it before starting deprotecting Fmoc group.

3.h. Final Wash

Add about 2mL of DCM into the vial, stir for 30 seconds and drain it. Repeat three times. Label and store it in a vial properly.

3.i. Cleavage

Mix TFA and DCM together to make 1% Trifluoracetic Acid (TFA) cleavage solution. Weight out certain amount of peptide and transfer it to the reaction vial. Add a stir bar into the vial. Then, add about 1mL of 1% TFA solution to the reaction vial, stir for 1~2 minutes, drain it and use a small flask to collect the solution. Repeat for at least five times. Rotavap the collected solution and test it in LCMS. Analyze the data.

3.j. Method development versus yield determination

In this project, the goal was primarily to devise a route to peptides that a student in our lab could utilize to effectively synthesize TMIAs. The specific stretch goal was to develop a method to produce product. It is important to recognize that there are two factors which makes yield determination difficult: a) at each step about 3-5 mg of resin was removed in order to verify each intermediate in the entire synthesis. The purity of this intermediate was verified by an extracted wavelength chromatogram at 265nm, which is the lambda max of the Fmoc group. In each case it was verified by total ion current in LC-MS that there were minimal, or no impurities related to any peptides, protected or deprotected amino acids or peptide combinations of those. b) at various points in the synthesis the remaining resin was divided into smaller portions in order to test out new procedures. As each portion becomes smaller, aliquots removed for analysis become more of a factor. For this reason, yield determination was not a priority in this research. Rather, relative purity of products after cleavage in each step and the final products were important. As described, yields can be determined by future students after SPPS methods are scaled up, in which case removal of micro-samples for assay by LC-MS is not a large factor.

Chapter 4. Puzzle pieces

4.a. Puzzle piece methodology

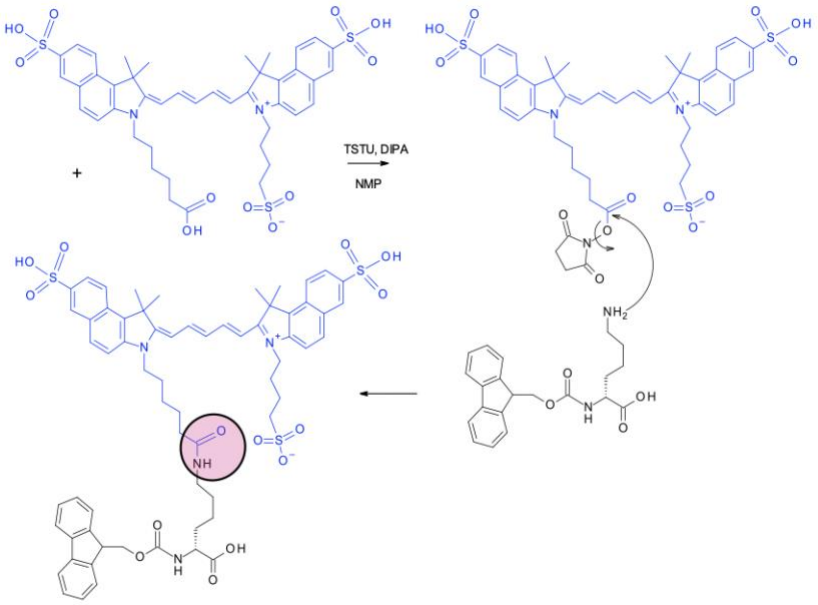
In order to create targeted molecular imaging agents (TMIAs) from the peptide targeting groups that are synthesizing, we will use makes use of the puzzle piece approach developed in our group. Puzzle pieces (also known as imaging modules) are synthesized by bonding imaging groups, such as near infrared (NIR) dyes to amino acids such as lysine, which have a reactive amine on the side chain. NIR dyes are useful in optical molecular imaging (OMI) methods including confocal fluorescence microscopy (CFM) and a variety of other *in-vitro* and *in-vivo* fluorescent imaging methods. [18]

In addition to the use of NIR dyes, we could use metal – chelate complexes such as gadolinium DOTA (Gadolinium (III) 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetate) that is used as a contrast agent in MRI. There are many MRI contrast agents based on Gd, [19] and examples of targeted Gd-based molecular imaging agents.[20] There are instances where chelated metals such as Gd-DOTA and radioactive metals such as Ga-DOTA have been put on peptides, but they are always put in after the peptide has been cleaved from the resin and most of the time the DOTA is protected during the synthesis with t-butyl groups which requires removal by harsh TFA.

To our knowledge the use of puzzle pieces to directly incorporate a NIR dye or a metal in SPPS has never been reported.

4.b. Cy5.5 (imaging module) puzzle piece

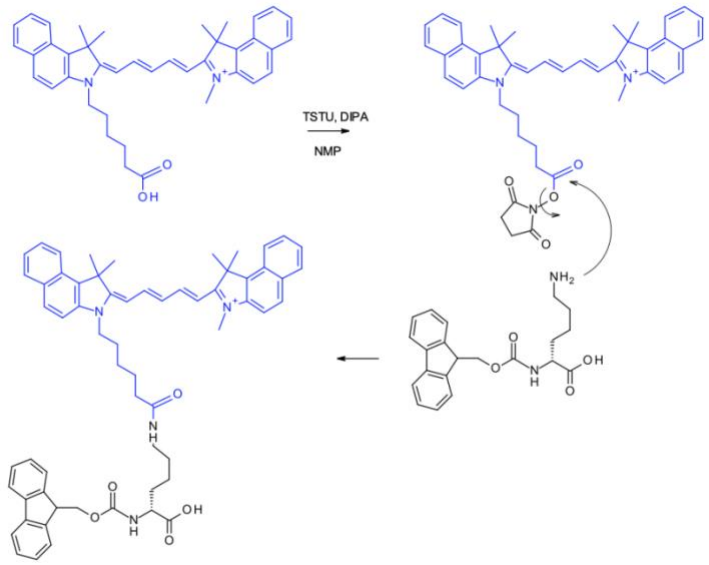
In our first attempt the imaging module (see Scheme 5) was synthesized utilizing a form of Cy5.5 that contained three sulfonates. The synthesis of this puzzle piece is shown in Scheme 5, where the dye is in blue and the Fmoc protected lysine amino acid in in black.



Scheme 5: Synthesis of Lysine Puzzle Piece with sulfonated Cy5.5

Unfortunately, in our test reactions with the tri-peptide model system shown above (Fmoc-Met-Ile-Phe-NH_2), the dye, was not able to be cleaved from the resin as indicated by the color. A number of different experiments involving stronger acid, base, different solvents and increased time were not effective. It was suspected that the sulfonic acid (sulfonate) groups on the dye irreversibly stuck to the resin. This was verified by an experiment in which the dye alone was mixed with the resin in various solvents, followed by an attempt to separate the dye by filtration. Regardless of conditions tried, the dye indeed stuck to the resin and could not be freed.

Based on this hypothesis, a lipophilic version of Cy5.5, containing no sulfonates, was purchased from Lumiprobe. The analogous puzzle piece was synthesized as shown in Scheme 6 and we were ultimately pleased to find that using the lipophilic version that the labelled peptide could be removed from the resin as shown in the next section.



Scheme 6: Synthesis of Lysine Puzzle Piece with non-sulfonated Cy5.5

4.c. Stability of imaging agents Cy5.5 and Gd-DOTA in dilute TFA

In prior synthesis in the lab, it was found that under acidic conditions, dyes would react with methanol (MeOH) to form a methyl adduct that was 14 amu higher in the mass spectra. For use in SPPS including the acidic condition of cleavage, conditions need to be investigated to test the stability of imaging agents including Cy5.5, IR800 and QC-1 which are the three main dyes of interest in our lab in order to eliminate this bi-product.

By a chance discovery, it was found that if dichloromethane (DCM) is added to the methanol as a co-solvent, the + 14 amu adduct from the extra methyl disappears. In initial

experiments, 50 % MeOH-DCM was found to protect the dye in acid, and in subsequent experiments even 20 % MeOH could also be used. After several experiments, Cy5.5 was stable in 1 % and 2 % TFA for at least three hours, and it even survived in 5% TFA for one hour. The table below summarizes the experiments used with the dyes in various solvents and conditions, monitoring the products by LC-MS.

Table 2: Summary of stability test of dyes (see Appendix I compounds 1-3 showing mass spectra and the observed color. Stable conditions are highlighted)

Stability Test							
Type of Dye	TFA conc. and cosolvent	Conc. (ug/mL)	Color	(m-2)/2 of Correct Dye Found	m/z of Correct Dye	m/z of Impurities	m/z Half Mass of Impurities Found
Cy5.5	0.01M TFA & MeOH	380	Blue			878.14	438.31
	0.005M TFA & MeOH	454	Blue			878.14	
	0.01M TFA & DCM & 20% MeOH	396	Light Blue	431.21	864.01		
	0.005M TFA & DCM & 20% MeOH	377	Light Blue	431.21	864.01		
IR800CW	0.01M TFA & MeOH	479	Green			1015.78	507.35
	0.005M TFA & MeOH	411	Green			1015.78	
	0.01M TFA & DCM & 50% MeOH	539	Green (grass)	1002.56	500.29		
	0.005M TFA & DCM & 50% MeOH	467	Green (grass)	1002.56	500.29		
QC-1	0.01M TFA & MeOH	443	Dark Green			1176.92	587.57
	0.005M TFA & MeOH	530	Dark Green			1176.92	
	0.01M TFA & DCM & 50% MeOH	453	Green (grass)	1079.85	539.18		
	0.005M TFA & DCM & 50% MeOH	486	Green (grass)	1079.85	539.18		

In previous research in the lab (unpublished results, Jessica Perez, project M.S., 2016), kinetic studies were performed of the chelated metal, Gd-DOTA, measured on the puzzle piece shown in Scheme 10. As shown in Figure 4, it was found that this chelate complex was stable in 0.1 M TFA in water for times up to one week. Based on these results, we may safely predict that the Gd-DOTA would be stable to the conditions of cleavage necessary for the Sieber resin, which we have determined is 1.0 % (0.088 M) or 2.0 % (0.175 M) TFA in dichloromethane. In addition, the cleavage time is 30 minutes which a small fraction of the week-long measurements in the previous study. However, this still more favorable than using pure TFA which is required in other SPPS resins such as Wang and Rink resins.

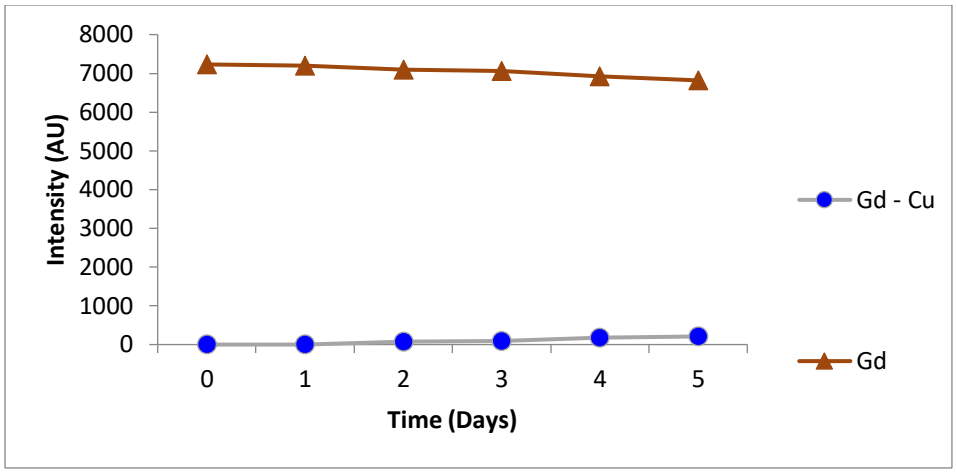
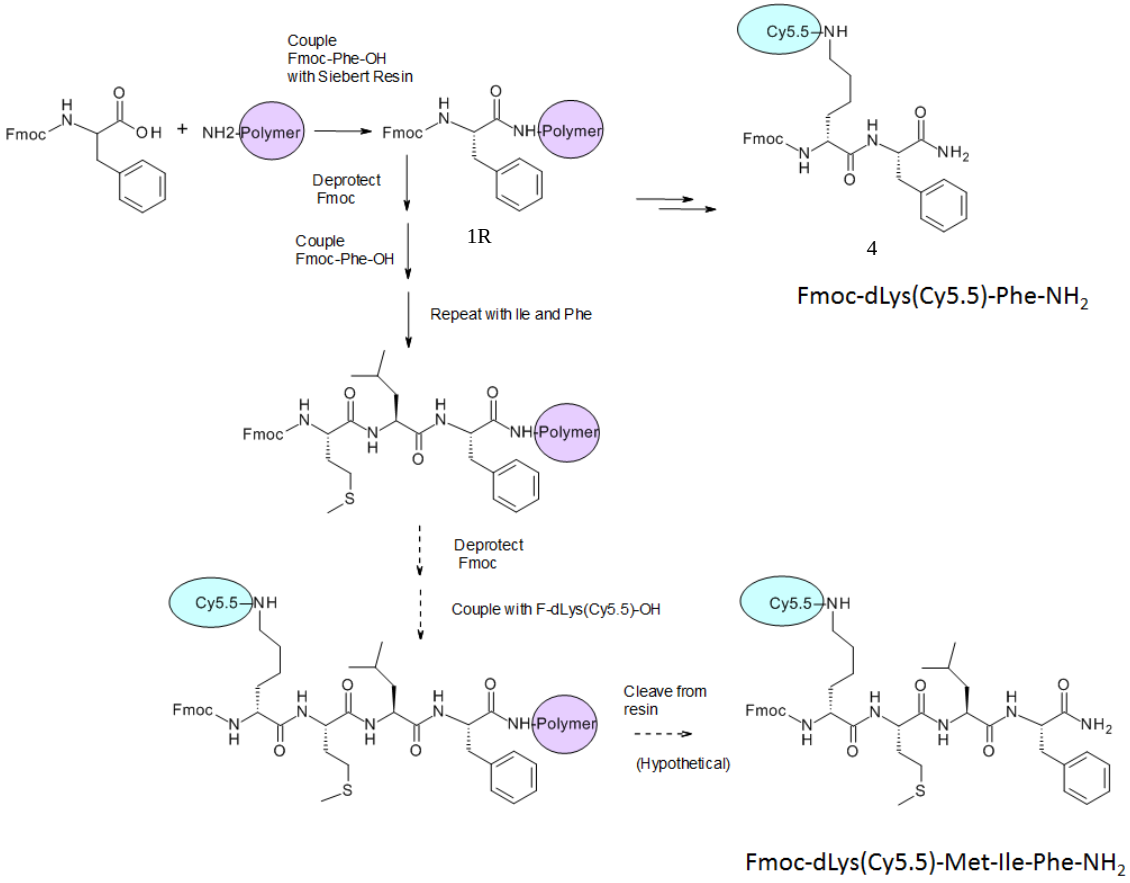


Figure 4: Graph showing the high stability of Gd-DOTA using the module Fmoc-Lys(Gd-DOTA)-NH_2. The displacement of Gd in 0.1M TFA was measured over several days, as well as the formation of Cu-DOTA in an excess of Cu+2 solution in the same solution. (Data from Jessica Perez, MS student, 2016.)

4.d. Synthesis tripeptides plus imaging modules Cy5.5

By using the optimized method for SPPS in Chapter 3, the puzzle piece was successfully coupled first to the mono-peptide shown in Scheme 7, followed by coupling this same imaging module to the tri-peptide. The products were verified by LC-MS which showed not only the correct mass of the product, but the expected absorption in the NIR region of the spectra at 684 nm of the same product.

While fluorescent dyes have been conjugated to peptides by solution methods, to our knowledge, after searching the literature, NIR dyes have never been incorporated as part of an SPPS followed by cleavage from the resin. Our puzzle piece method has enabled this goal and this success paved the way for future syntheses involving larger peptides.

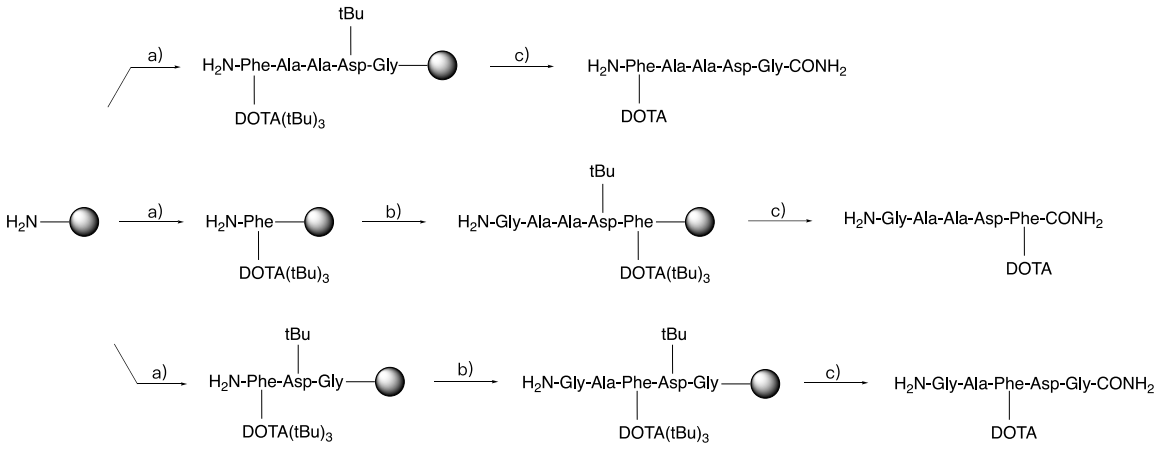


Scheme 7: Solid Phase Synthesis of Tripeptide with imaging agents: Cy5.5

4.e. Gd-DOTA puzzle pieces

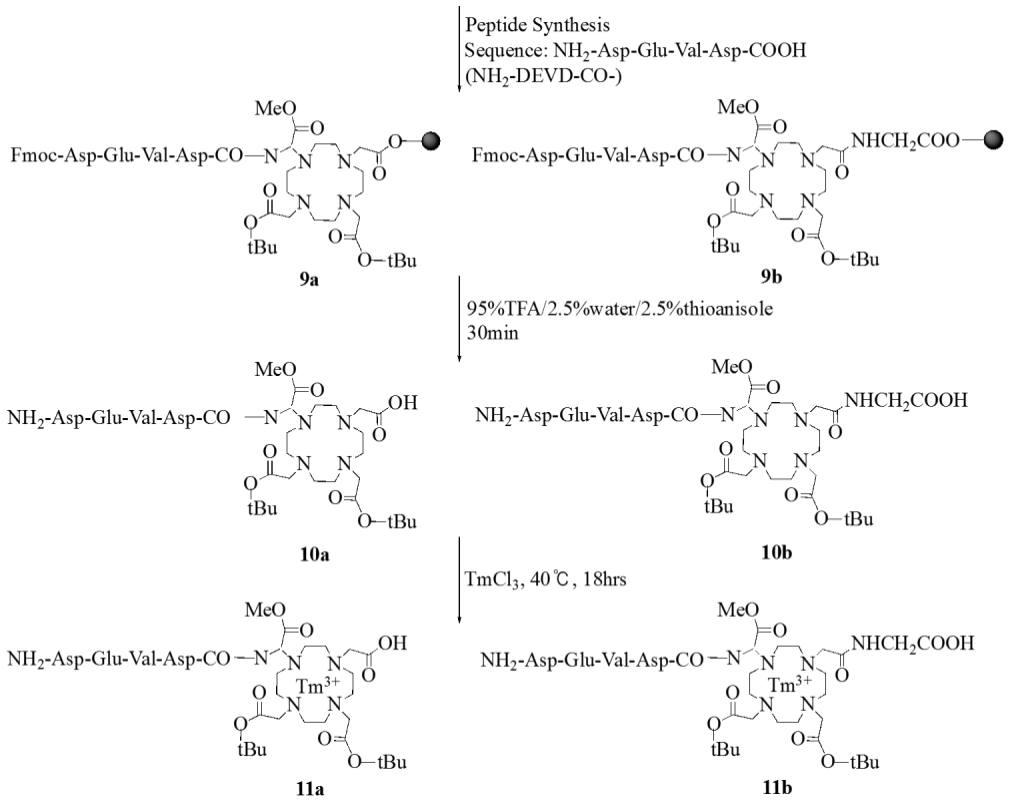
The next goal was to apply the puzzle piece method to the SPPS of a tri-peptide containing the metal Gd as its DOTA chelated complex.

Peptides containing Gd-DOTA on a lysine have been assembled in the past. In a pioneering study, Sherry and co-workers have brought in a lysine or a phenylalanine containing a protected DOTA group as its tri-t-butyl ester as shown in Scheme 8.[21] In that case, the tri-t-butyl group was removed as part of the acid catalyzed cleavage process, and the final DOTA containing peptide was metalized in the last step.



Scheme 8: Synthesis by Prof. Dean Sherry's group (world expert in chelated metals)[21]

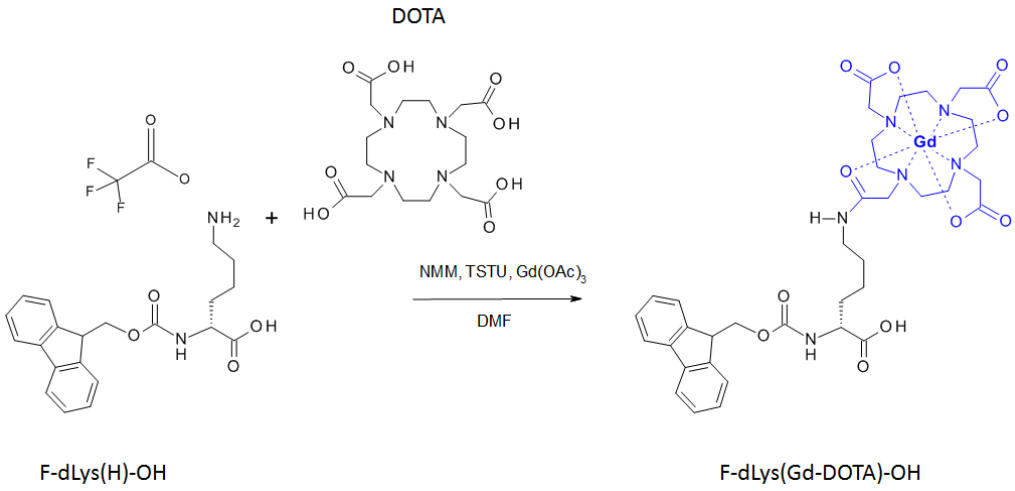
In a second related article, a similar approach was reported by Yoo and Pagel, a di-t-butyl protected DOTA was fastened directly to the SPPS resin via a linker and the peptide was built onto that.[22] This approach was clearly different from the approach by Sherry who incorporated the DOTA mid-way through the synthesis. In either approach, strong 100 % TFA must be utilized to protect t-butyl groups from DOTA. In our group it was previously determined that lower concentrations of TFA are not effective because when t-butyl groups are on DOTA, they are more stable than normal t-butyl esters. This would prevent the use of dyes in the SPPS which require the use of lower TFA concentrations as described below.



Scheme 9: Synthesis using Di-t-butyl protected DOTA on SPPS by Yoo and Pagel[22]

In contrast to the above approach, the use of puzzle pieces to directly incorporate a metal-chelate complex such as in SPPS has never been reported to our knowledge. One advantage of this approach is that there is no need for harsh treatment by pure TFA which is necessary to remove the t-butyl groups from DOTA. In our approach 1% TFA is used for a variety of reasons including the acid-sensitivity of most dyes. So, this approach in which the metal is added early in the synthesis is unique and provides several advantages.

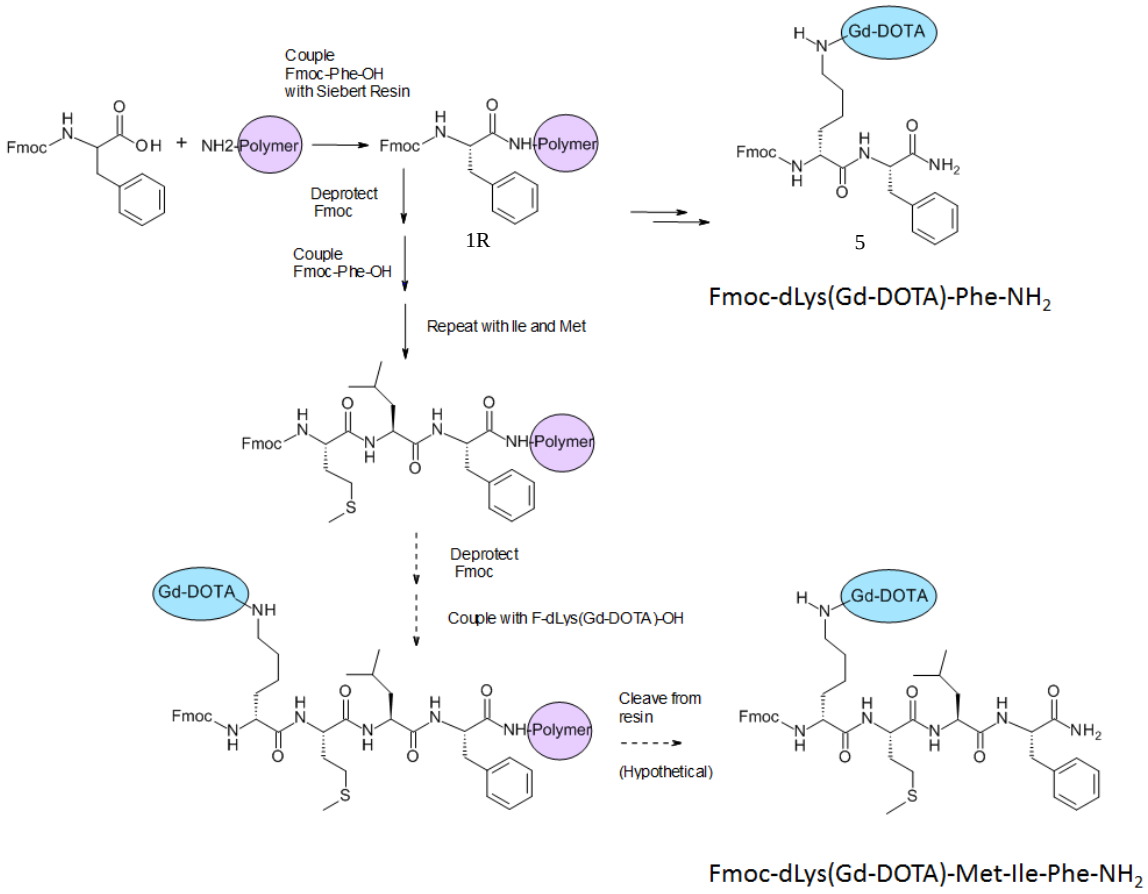
The synthesis of the puzzle piece containing the Gd-DOTA is shown in Scheme 10. The procedure is a "one pot synthesis" developed in our lab which gives the module directly and was an improvement over a three steps procedure involving a protection-deprotection scheme (not shown).



Scheme 10: Synthesis of Lysine Puzzle Piece with Gd-DOTA

4.f. Synthesis of tri-peptides plus Gd-DOTA

By using the optimized method for SPPS in Chapter 3, the puzzle piece containing the Gd-DOTA was successfully coupled first to the mono-peptide shown in Scheme 11, followed by coupling to the tri-peptide. This success paved the way for future syntheses involving larger peptides containing Gd-DOTA.



Scheme 11: Synthesis of Tripeptide with imaging agents for MRI: Gd-DOTA

Chapter 5. Met-enkephalins

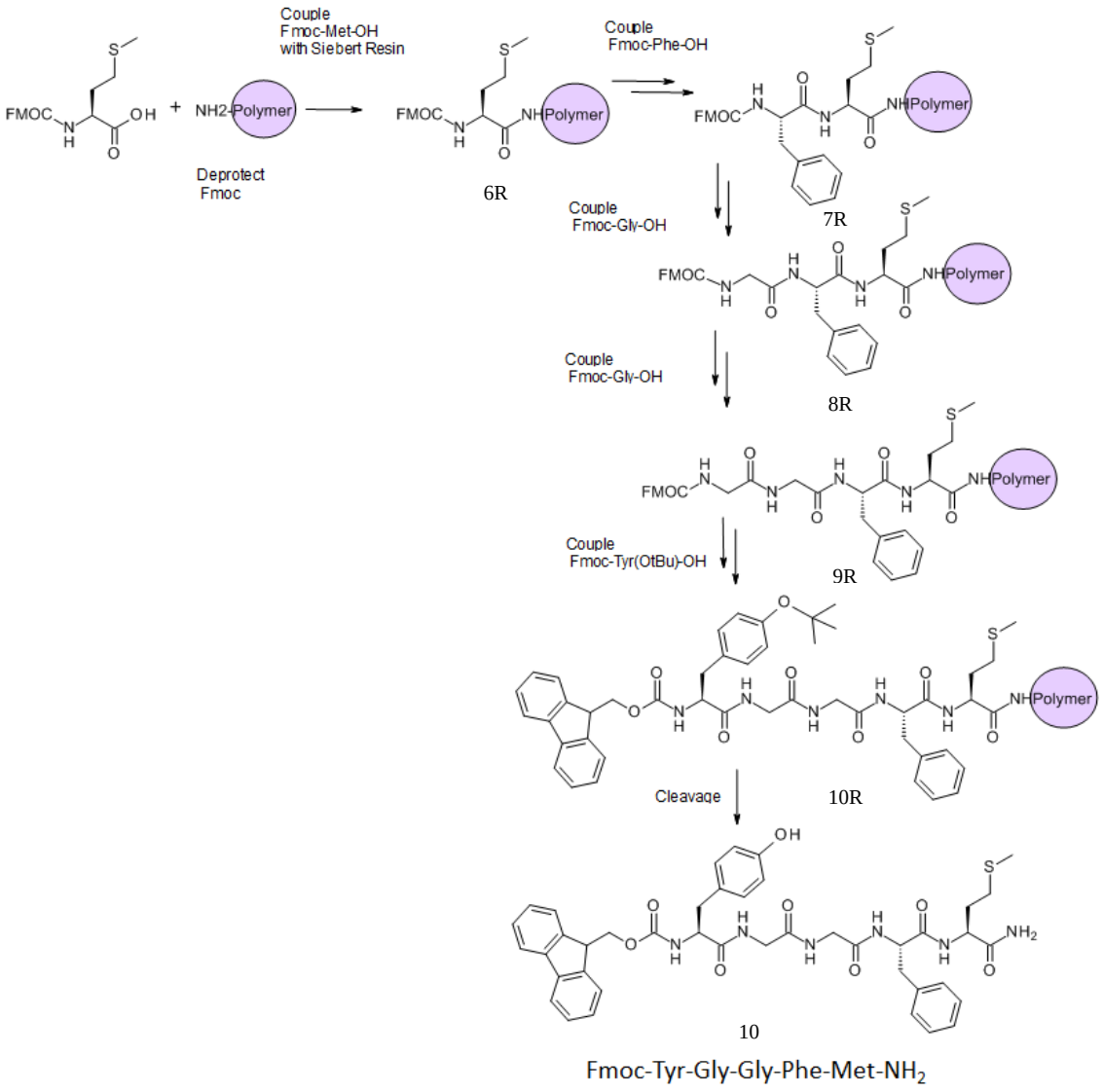
5.a. The choice of Met-enkephalins as a model system

Met-enkephalins are short peptides which are the active site of endorphins which are larger proteins that contain the pentapeptide somewhere on their sequence. The discovery of enkephalins followed research findings which linked morphine and heroin and other opiates to a natural receptor site. This receptor site was part of a protein system called endorphin, which is a natural protein in the body (animal or human). The binding of natural or synthetic opiates (from poppy flowers) can also be a pain killer (and produce a "high"). So, after the endorphins were found, researchers reduced the size of the peptide until they found the minimal active sequence - which were two enkephalins, Met-enkephalin and Leu-enkephalin.

Instead of using just a random peptide such as the tri-peptide utilized in our first optimization efforts, we chose to synthesize Met-enkephalin and label it with both of our puzzle piece imaging modules because it is a "biologically relevant" or "biologically important" peptide. It was also anticipated that dye and Gd – based TMIAs based on enkephalin may be novel and may be useful in imaging opioid receptors.

5.b. Synthesis of Met-enkephalin

The preparation of the pentapeptides, Met-enkephalin by the following method, carried out on Sieber (Xanthenyl) resin, was successful. This paved the way for bringing in the puzzle pieces containing Cy5.5 and Gd-DOTA.

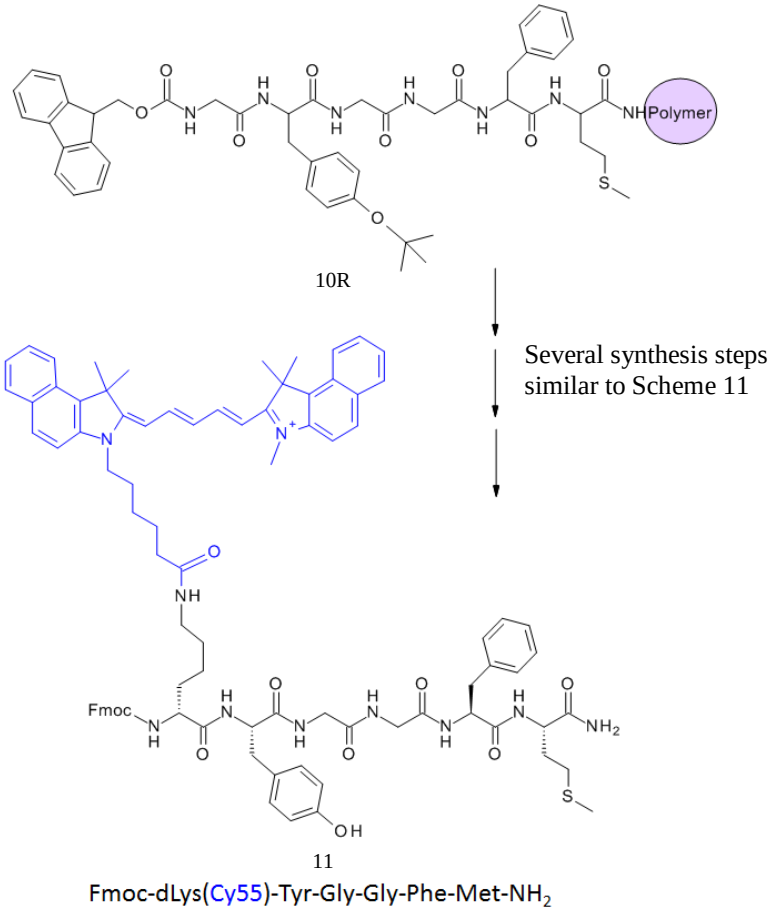


Scheme 12: Synthesis of Met-enkephalin

5.c. Cy5.5 Met-enkephalin

The method applied to the synthesis of the Cy5.5 labelled tri-peptide as designed in chapter 4d was utilized to synthesis Fmoc-dLys(Cy5.5)-NH_2 by the method shown in Scheme 13. In the case of enkephalin, it is necessary to bring in the tyrosine in its t-butyl ether protected form. Our design is based on cleavage from the Sieber resin in 1% TFA

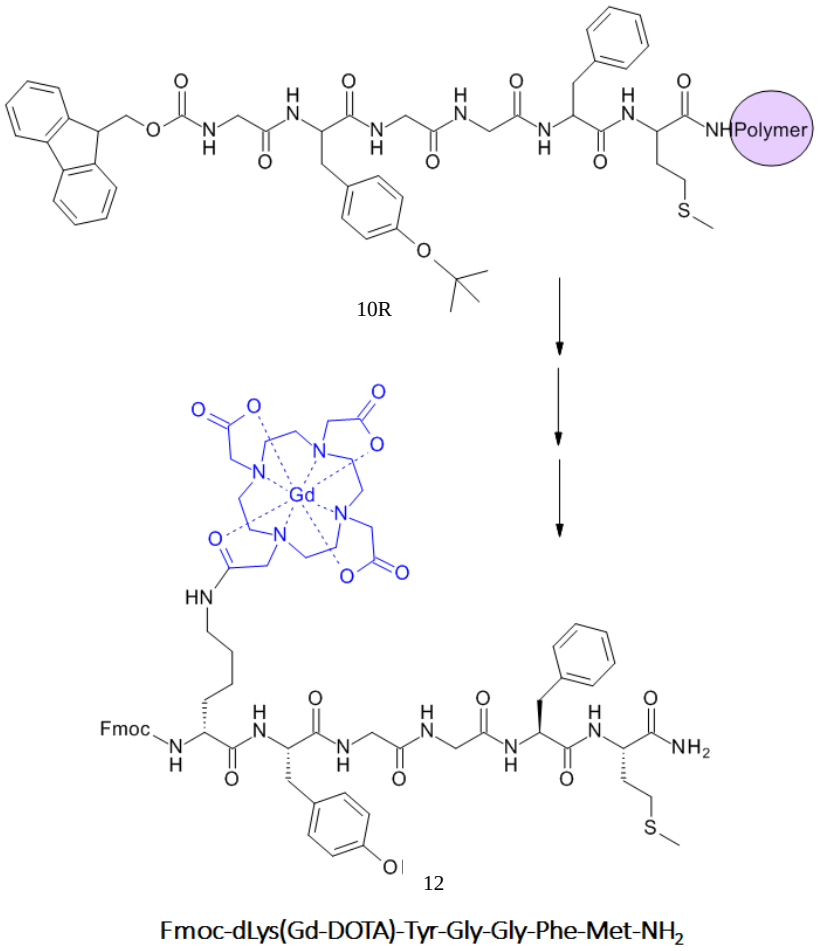
and this is fortunate, as t-butyl ethers may be removed by very mild treatment, such as 1 % TFA. In addition to this, the NIR dye Cy5.5 is stable to 1 % TFA.



Scheme 13: Synthesis of Fmoc-dLys(Cy5.5)-Met-enkephalin (11)

5.d. Gd-DOTA Met-enkephalin

The preparation of the Gd-DOTA Met-enkephalin containing the Gd-DOTA was successful. Likewise, as expected from the earlier kinetic studies by Anne Marie Sweeney and Jessica Perez in our group the Gd-DOTA complex is stable in the mild 1 % cleavage solution.



Scheme 14: Synthesis of Fmoc-dLys(Gd-DOTA)-Met-enkephalin (12)

Chapter 6. Breast cancer peptides

6.a. Breast cancer targeting peptide 18-4

The stretch goal of this research was to apply the SPPS methods we developed to the synthesis of an actual targeted imaging agent for cancer. A beautiful example of a targeting peptide useful for imaging breast cancer was recently reported by Dr. Kaur.[12–14]

In her studies, Kaur began with a previously developed breast cancer targeting peptide p160 (12-mer) and prepared an enzymatically stable analogue 18-4 (10-mer) of this peptide, shown in Figure 5, that showed remarkable potential for targeted breast cancer drug delivery. The peptide was shown to target MDA-MB-231 cells in vitro by fluorescein labelling using CFM then extended to the delivery of drugs including streptomycin in animal studies.

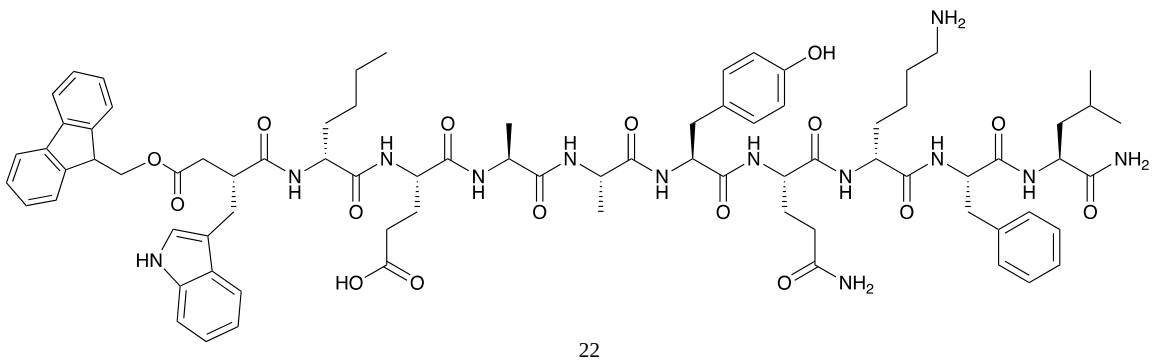


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Figure 5: Structure of Breast cancer peptide

Following this peptide, a cyclic peptide, "peptide 7", was prepared by Dr. Kaur. Animal studies using mice with orthotopic breast MDA-MB-231 tumors showed that the cyclic peptide selectively accumulates in tumor and is rapidly cleared from all other organs except kidneys and liver. While the cyclic peptide may be somewhat better, Dr. Kaur advised us that the linear version would be suitable for our studies and would show the same kind of results, and that it would be far simpler to synthesize.

In initial work by others in our group, a 20 mg sample of peptide 18-4 was sent as a gift by Dr. Kaur to our lab for labelling purposes. A very small amount of this peptide was labelled with Cy5.5 itself (not the puzzle piece) at the third amino acid (d-lysine, or k) to form the TMIA we called "M1" shown in Figure 6 where the peptide is shown using one-letter abbreviations for the amino acids, shown in red.

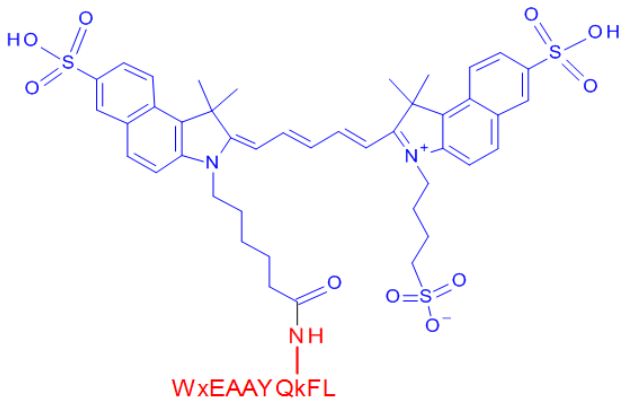

Figure 6: TMIA "M1", peptide 18-4 labelled on the 3-lysine side chain with Cy5.5

Our collaborators in the School of Life Sciences, Dr. Evans and her group, were able to grow MDA-MD-231 breast cancer cells and stain them with 0.1 μM of agent "M1" and stained with NucBlu which show the nuclei of cells only (in blue).

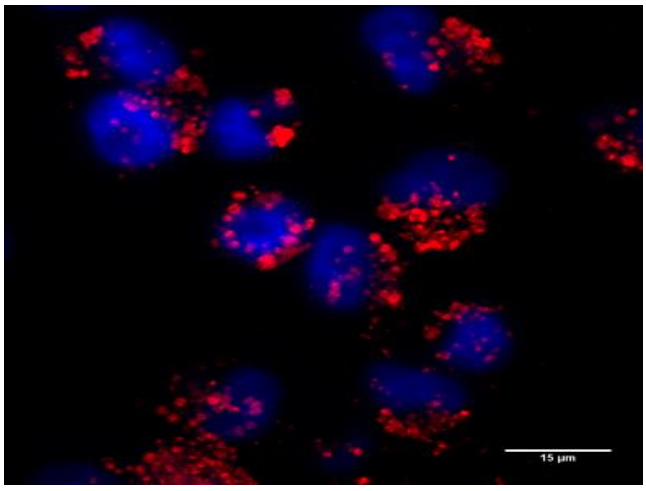

Figure 7: MDA-MD-231 BrCa cells stained with TMIA "M1" and NucBlu in collaboration with Dr. Irene Evans, GSOLS, RIT.

After obtaining these preliminary results, which mirrored results obtained in the Kaur group (using fluorescein as the NIR dye) we were encouraged to pursue the synthesis of the peptide ourselves by the SPPS method described above. This was also a good time to do this as we had run out of the gifted peptide, and we were having considerable difficulty in reproducing the initial synthesis of M1.

6.b. SPPS of breast cancer targeting peptide 18-4

The peptide was successfully synthesized by using the methodology developed as described in Chapter 3. Each step was carefully monitored by cleaving a tiny amount of the resin with 1 % TFA and assaying the trace residue by LC-MS. The recipe works well and each coupling and each deprotection step proceeded perfectly as shown by LC-MS, described in Chapters 2c, 3e and 8 and shown in the LC-MS data in Appendix I. In most cases the acid-sensitive protecting groups were removed but in some cases a mixture of protected and deprotected intermediates were observed. The Mtt on d-lysine is removed rapidly, but t-butyl on tyrosine and t-butyl on glutamic acid are partially removed. The Boc group on tryptophan is difficult to remove and requires harsher conditions. In work carried on in the lab, unprotected tryptophan is being used.

The synthesis is represented in the following table which shows each intermediate in its protected and deprotected forms, along with the calculated full mass and half mass. The results from the LC-MS are shown in Appendix I (compounds 12-23), showing that in each case the major product was relatively pure and showed the correct mass or half mass. In addition, a high-resolution mass spectra (HRMS) was obtained on the final peptide and the results proved that the structure was peptide 18-4 in its protected form, Fmoc-Trp(Boc)-

d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (HRMS results: MW 1701.9296 theory vs 1701.9264 for $M+H^+$, deviation of 1.9 ppm).

Table 3: Molecular Weight of Peptides

Compounds	Molecular Weight (g/mol)	Half Mass (g/mol)
Fmoc-Leu-NH_2	352.2	177.1
Fmoc-Phe-NH_2	386.2	194.1
Fmoc-Phe-Leu-NH_2	499.2	250.6
Fmoc-d-Lys(Mtt)-Phe-Leu-NH_2	883.5	442.8
Fmoc-d-Lys-Phe-Leu-NH_2	627.3	314.7
Fmoc-Gln-d-Lys-Phe-Leu-NH_2	755.4	378.7
Fmoc-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	974.5	488.3
Fmoc-Tyr-Gln-d-Lys-Phe-Leu-NH_2	918.5	460.3
Fmoc-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1045.6	523.8
Fmoc-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	989.5	495.8
Fmoc-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1116.6	559.3
Fmoc-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1060.5	531.3
Fmoc-Glu(OtBu)-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1245.6	623.8
Fmoc-Glu-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1245.6	623.8
Fmoc-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1301.7	651.9
Fmoc-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1189.6	595.8
Fmoc-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1302.7	652.4
Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1358.7	680.4
Fmoc-d-Nle-Glu-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1358.7	680.4
Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1414.8	708.4
Fmoc-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1488.7	745.4
Fmoc-Trp-d-Nle-Glu(OtBu)-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1544.8	773.4
Fmoc-Trp-d-Nle-Glu-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1544.8	773.4
Fmoc-Trp-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1600.9	801.5
Fmoc-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1700.9	851.5

It is important to note that all of the peptides in this sequence are very lipophilic. This causes them to stick onto the C-18 column creating wide peaks and requiring the use of increasing percentages of organic solvent (acetonitrile) as the chain increases. We were

able to chromatograph the intermediates up to the nona-peptide (gradient 80-100, but when we added the tenth amino acid, tryptophan we could no longer use a column and switched to "loop injections".

6.c. Cy 5.5 labelled breast cancer Fmoc-protected targeting peptide 18-4

Utilizing the SPPS resin that contained the fully protected deca-peptide 18-4, the non-sulfonated Cy5.5 puzzle piece described in Chapter 4b was coupled onto the resin to yield the final product "M2" in its protected form. The entire resin was then cleaved with 1 % TFA to yield an analytical sample of the TMIA final product as partially protected peptide Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH2, (HRMS results: MW 1198.1691 theory vs 1198.1732 for $(M+2H^+)/2$, deviation of 3.42 ppm).

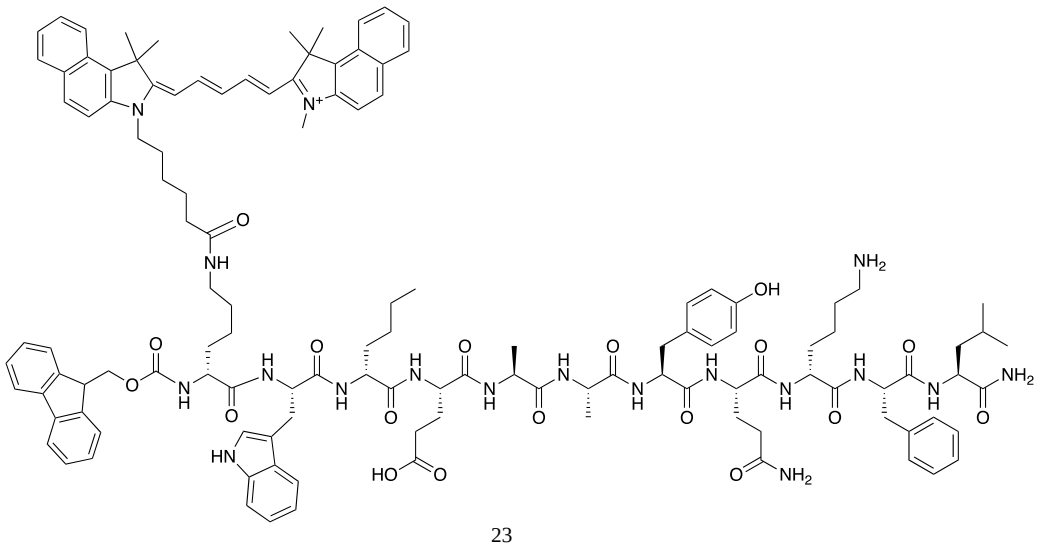

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Figure 8: Structure of Breast cancer peptide with non-sulfonated Cy5.5

Table 4: Molecular Weight of Peptides

Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	2394.3381	1198.17
Fmoc-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	2182.1610	1092.08

6.d. Cy 5.5 labelled breast cancer targeting peptide 18-4

Utilizing the SPPS resin that contained the fully protected deca-peptide 18-4, the non-sulfonated Cy5.5 puzzle piece described in Chapter 4b was coupled onto the resin to yield the final product "M2" in its protected form. Fmoc group has been taken off before cleavage. The entire resin was then cleaved with 1 % TFA to yield an analytical sample of the TMIA final product as partially protected peptide NH_2-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2, (HRMS results: MW 981.05194 theory vs 981.0515 for $(M+2H^+)/2$, deviation of 0.448 ppm).

A 100 μM solution of this compound was given to Dr. Evans group (GSOLS) for testing in BrCa cells by CFM.

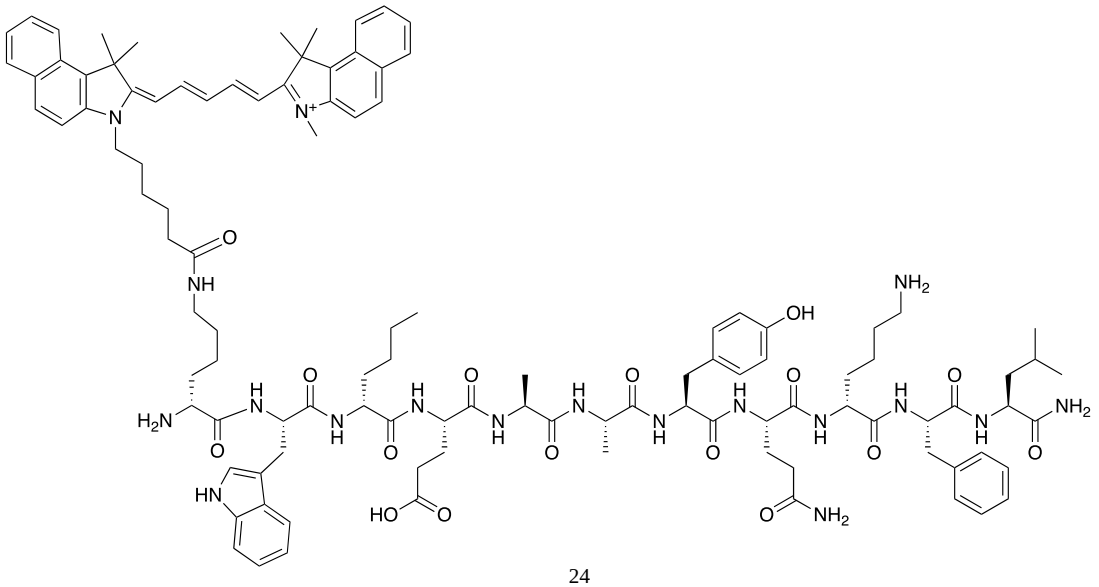

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Figure 9: Structure of Breast cancer peptide with non-sulfonated Cy5.5 (No Fmoc protecting group)

Figure 10: Molecular Weight of Peptides

NH_2-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1960.08800	981.05194
Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	2182.15990	1092.08589

6.e. Gd-DOTA labelled breast cancer targeting peptide 18-4

Utilizing the SPPS resin that contained the fully protected deca-peptide 18-4, the Gd-DOTA puzzle piece, described in Chapter 4e was coupled onto the resin to yield the final product "G2" in its protected form. The entire resin was then cleaved with 1 % TFA to yield an analytical sample of the final TMIA product as partially protected peptide Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2, (HRMS results: MW 1186.05632 theory vs 1186.0563 for $(M+2H^+)/2$, deviation of 0.02 ppm.)

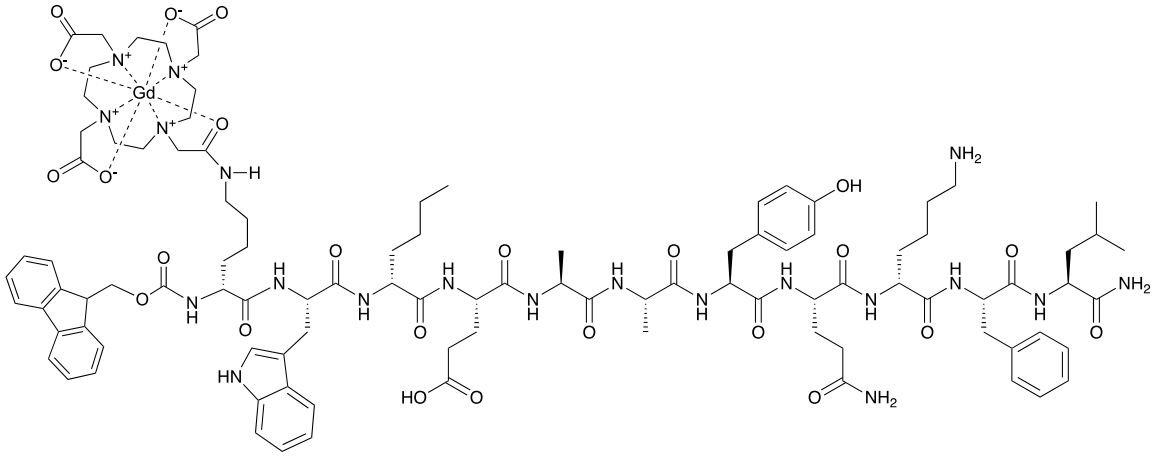

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Figure 11: Structure of Breast cancer peptide with metal-chelate complex Gd-DOTA

Table 5: Molecular Weight of Peptides

Fmoc-d-Lys(Gd-DOTA)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	2167.9199	1084.9600
Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	2370.0970	1186.0563

6.f. Confocal Fluorescence Microscopy of Breast Cancer Cells Targeted with Cy5.5 labelled 18-4

In the final weeks of research Matt Law resynthesized the Cy5.5 labelled breast cancer peptide 18-4 in the totally deprotected form using the method described here. Then our collaborators in Biology (Dr. Irene Evans and her student Josh Evans) were successful in culturing MDA-MB-231 breast cancer cells (triple negative type) *in vitro*, followed by staining these with 10 μM solutions of the Cy5.5-18-4 TMIA.

The following images were obtained using the Zeiss CFM in the School of Life Sciences at RIT. This demonstrates that the approach of labelling 18-4 by placing the Cy5.5 lysine puzzle piece (module) on the N-terminus of the peptide was highly successful.

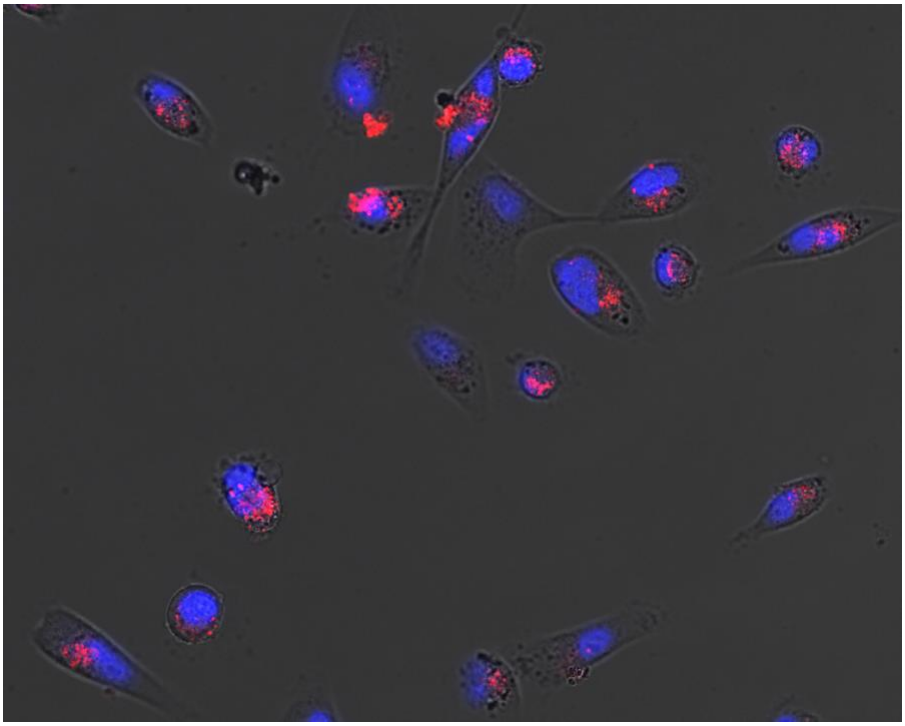

Figure 12: CFM image of MDA-MB-231 breast cancer cells stained with Cy5.5-18-4 TMIA, blue is the nucleus of each cell (NucBlu), red is TMIA

Chapter 7. Conclusion

A method has been developed for the solid phase synthesis of peptides using the Sieber resin. This method has been tested by the synthesis of a mono-peptide (Fmoc-Phe-NH_2), di-peptide (Fmoc-Ile-Phe-NH_2) and tri-peptide (Fmoc-Met-Ile-Phe-NH_2) followed by successfully coupling two types of imaging modules (puzzle pieces) separately onto the mono-peptide (Fmoc-Phe-NH_2). The imaging modules consisted of an Fmoc protected lysine coupled to a NIR dye, Cy5.5, on the side chain nitrogen, or a Gd-DOTA coupled to a lysine in the same manner.

After optimizing the method for synthesizing peptides, the method was documented in the form of a recipe, then applied to the synthesis of a bioactive penta-peptide, Met-enkephalin, followed by similarly attaching the two puzzle pieces containing Cy5.5 or Gd-DOTA to this peptide.

A stretch goal was then achieved by synthesis of a breast cancer targeted peptide 18-4, a deca-peptide reported by Dr. Kaur of Chapman University. Puzzle pieces or modules containing a lipophilic Cy5.5 dye and Gd-DOTA were also coupled successfully to the resin containing the protected form of 18-4, followed by cleavage with dilute 1 % TFA to yield the partially and fully deprotected TMIAs.

The value of this work was to provide the molecular imaging lab (MIL) at RIT with a powerful method to synthesize not only targeting peptides by SPPS, peptides by SPPS, and to synthesize targeted molecular imaging agents (TMIAs) that will be useful in the detection of breast cancer. In the larger scope, these methods may be widely used to synthesize peptide-based TMIAs for detecting a wide variety of cancers by molecular imaging methods.

Chapter 8. Experimental Procedures

Materials and Methods

Chemicals were purchased from VWR (Radnor, PA), Sigma Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), TCI (Tokyo, Japan), and Acros Organics (Morris Plains, NY), and were used as received unless otherwise stated. All were HPLC or American Chemical Society grade. Amino acid starting materials and Xanthenyl linker resin were purchased from Bachem (Bubendorf, Switzerland), and Chem-Impex Int'l Inc. (Wood Dale, IL).

The HPLC instrument used was an Agilent 1100 with Diode Array Detector and for LC-MS a Waters 2695 Alliance HPLC with a Waters 2998 Diode Array Detector and a Waters 3100 SQ Mass Spectrometer was used. For HPLC the columns used were: an Agilent XDB C18 column, with dimensions of 3 mm x 100 mm or a Waters XBridge C18 column 50 mm x 3 mm and 3µ particle size. Mass spectra from this instrument were recorded at unit resolution with positive and negative switching mode at 35 or 50 V cone voltages. The flow rate for HPLC-MS was 0.5 mL/min. All aqueous mobile phases for HPLC are 0.1M ammonium acetate unless otherwise noted.

Preparative HPLC (prep-HPLC) was carried out with a Waters 600E system controller, and Waters 600 multi-solvent delivery system using a 30 mL/min flow rate. For SPE purification, a 20 g C-18 Sep-pack Varian Mega Bond Elut (20CC/5GRM) SPE cartridge was utilized for DCL (7) and Gd/La DOTA compounds and the DOTA transmetalations utilized a Varian Bond Elut (C18, 3CC/1GRM) SPE cartridge.

Chromatography gradients for HPLC, prep-HPLC, and SPE are noted as (method X: Y, t) where X is the organic mobile phase being either acetonitrile (ACN) or methanol (MeOH), Y is the starting percentage or overall range of the organic mobile phase, and t is the overall time in minutes of the gradient except for SPE where this is not applicable. For example, method (ACN: 60, 8min) is a gradient that is initially 60% ACN and 40% H_2O, but gradually transitions to 100% ACN and 0% H_2O in 8 minutes. Method (MeOH: 20-70, 8min) is a gradient that starts at 20% MeOH and 80% H_2O, and over 8 minutes the gradient transitions to 70% MeOH and 30% H_2O.

All aqueous mobile phases for prep-HPLC are 0.1M ammonium acetate unless otherwise noted. Aqueous mobile phases for SPE are not buffered unless otherwise noted. The SPE cartridges were conditioned with their respective organic solvent, then pure DI H2O, then equilibrated with the initial gradient concentration. Gradients were performed in 5% increments with 3-10 mL fractions each unless otherwise noted. Silica gel for flash chromatography was purchased from Alfa Aesar and was 70-230 mesh. For DOTA compounds, a Keystone Scientific, Inc. Hyperprep C18 BDS 250mm x 20 mm column with an 8µ particle size was used for prep-HPLC.

High resolution mass spectra (HRMS) were obtained on a Waters Synapt G2Si (School of Chemical Sciences, University of Illinois at Urbana-Champaign) using the

following parameters: Flow injection at flow rate of 0.1 ml/min, H2O/ACN/0.1% Formic Acid, positive and negative mode ESI, Cone voltage =25 V, capillary voltage = 3.0, ion source temperature = 100°C, desolvation temperature =180°C, nebulizing gas (N_2) flow = 200 L/h, cone gas (N_2) flow = 5L/h.

Synthetic Procedures

Fmoc-Phe-NH_2 (1). Xanthenyl linker resin (100.0 mg, 29.1 μmol) was pre-swelled in DMF (2 mL). After draining, mixture of 5% Piperazine, 2% DBU and 93% DMF (3 × 2 mL) were applied to the resin. DMF (3 × 2mL) was added to wash the beads. Fmoc-Phe-OH (22.5 mg, 58.2 μmol) was dissolved in 2 mL DMF, followed by addition of DIEA (49.7 μL, 29.1 μmol). HATU (21.0 mg, 55.3 μmol) was dissolved separately in 2 mL DMF added to the previous mixture and shaken. Then this solution was added to the resin within 1 minute. After being stirring for about 45 minutes at room temperature, the reaction solution was drained and washed with pure DMF (3 × 2 mL) and DCM (2 × 2 mL). A small amount of the resultant resin (1R) (10 μg), was removed and cleaved with 1% TFA in DCM (5 × 1 mL) for 1-2 min, drained through the vessel frit. The eluent was concentrated on a rotovap, dissolved in 0.5 mL methanol and assayed by LC-MS. LC-MS (LR, ESI) = Calcd. for $C_{24}H_{22}N_2O_3$: 386.444 (m/z), found: 387.28 $[M+H]^+$.

Fmoc-Ile-Phe-NH_2 (2). Resin 1R was treated by the same procedure as for (1) above using Fmoc-Ile-OH (20.6 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (2R) also by the same procedure as above to yield of Fmoc-Ile-Phe-NH_2 (2). LC-MS (LR, ESI) = Calcd. for $C_{30}H_{33}N_3O_4$: 499.602 (m/z), found: 500.45 $[M+H]^+$.

Fmoc-Met-Ile-Phe-NH_2 (3). Resin 2R was treated by the same procedure as for (1) above using Fmoc-Met-OH (21.6 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin also by the same procedure as above to yield of Fmoc-Met-Ile-Phe-NH_2 (3). LC-MS (LR, ESI) = Calcd. for $C_{35}H_{42}N_4O_5S$: 630.288 (m/z), found: 631.49 $[M+H]^+$.

Fmoc-d-Lys(Cy5.5)-Phe-NH_2 (4). Take a small amount of resin 1R (10 μg), treated by the same procedure as for (1) above using Fmoc-d-Lys(Cy5.5)-NH_2 (5.43 mg, 5.82 μmol), followed by cleavage of the resultant resin also by the same procedure as above to yield of Fmoc-d-Lys(Cy5.5)-Phe-NH_2 (4).The yield was 4.6 mg (61.3%). LC-MS (LR, ESI) = Calcd. for $C_{70}H_{75}N_6O_5$: 1080.384 (m/z), found: 1080 $[M+H]^+$. LC-MS (HR, ESI) = Calcd. for $C_{70}H_{75}N_6O_5$: 1079.57989, found: 1080.5801 $[M+H]^+$.

Fmoc-d-Lys(Gd-DOTA)-Phe-NH_2 (5). Take a small amount of resin 1R (10 μg), treated by the same procedure as for (1) above using Fmoc-d-Lys(Gd-DOTA)-NH_2 (5.3 mg, 5.82 μmol), followed by cleavage of the resultant resin also by the same procedure as above to yield of Fmoc-d-Lys(Gd-DOTA)-Phe-NH_2 (5). The yield was 15.7 mg (87.2%). LC-MS (LR, ESI) = Calcd. for $C_{46}H_{57}GdN_8O_{11}$: 1055.246 (m/z), found: 1056.32 $[M+H]^+$. LC-MS (HR, ESI) = Calcd. for $C_{46}H_{57}GdN_8O_{11}$: 1055.33878, found: 1056.3452 $[M+H]^+$.

Fmoc-Met-NH_2 (6). Xanthenyl linker resin (100.0 mg, 29.1 μmol) was pre-swelled in DMF (2 mL). After draining, mixture of 5% Piperazine, 2% DBU and 93% DMF (3 × 2 mL) were applied to the resin. DMF (3 × 2mL) was added to wash the beads. Fmoc-Met-OH (21.6 mg, 58.2 μmol) was dissolved in 2 mL DMF, followed by addition of DIEA (49.7 μL, 29.1 μmol). HATU (21.0 mg, 55.3 μmol) was dissolved separately in 2 mL DMF added to the previous mixture and shaken. Then this solution was added to the resin within 1 minute. After being stirring for about 45 minutes at room temperature, the reaction solution was drained and washed with pure DMF (3 × 2 mL) and DCM (2 × 2 mL). A small amount of the resultant resin (3R) (10 μg), was removed and cleaved with 1% TFA in DCM (5 × 1 mL) for 1-2 min, drained through the vessel frit. The eluent was concentrated on a rotovap, dissolved in 0.5 mL methanol and assayed by LC-MS. This resin was taken directly on to the next step without characterization.

Fmoc-Phe-Met-NH_2 (7). Resin 3R was treated by the same procedure as for (4) above using Fmoc-Phe-OH (22.5 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (4R) also by the same procedure as above to yield of Fmoc-Phe-Met-NH_2 (7). This resin was taken directly on to the next step without characterization.

Fmoc-Gly-Phe-Met-NH_2 (8). Resin 4R was treated by the same procedure as for (4) above using Fmoc-Gly-OH (17.3 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (5R) also by the same procedure as above to yield of Fmoc-Gly-Phe-Met-NH_2 (8). This resin was taken directly on to the next step without characterization.

Fmoc-Gly-Gly-Phe-Met-NH_2 (9). Resin 5R was treated by the same procedure as for (4) above using Fmoc-Gly-OH (17.3 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (6R) also by the same procedure as above to yield of Fmoc-Gly-Gly-Phe-Met-NH_2 (9). This resin was taken directly on to the next step without characterization.

Fmoc-Tyr-Gly-Gly-Phe-Met-NH_2 (10). Resin 6R was treated by the same procedure as for (4) above using Fmoc-Tyr(tBu)-OH (26.7 mg, 58.2 μmol), followed by cleavage of a

small amount the resultant resin (7R) also by the same procedure as above to yield of Fmoc-Tyr-Gly-Gly-Phe-Met-NH_2 (10). LC-MS (LR, ESI) = Calcd. for $C_{42}H_{46}N_6O_8S$: 794.916 (m/z), found: 795.31 $[M+H]^+$. LC-MS (HR, ESI) = Calcd. for $C_{42}H_{46}N_6O_8S$: 794.30978, found: 795.3162 $[M+H]^+$.

Fmoc-d-Lys(Cy5.5)-Tyr(tBu)-Gly-Gly-Phe-Met-NH_2 (11). Resin 7R (8 mg, ca. 10 % of the prior yield of 7R) was treated by the same procedure as for (4) above using Fmoc-d-Lys(Cy5.5)-NH_2 (5.43 mg, 5.82 μmol), followed by cleavage of the resultant resin also by the same procedure as above to yield of Fmoc-d-Lys(Cy5.5)-Tyr(tBu)-Gly-Gly-Phe-Met-NH_2 (11). The yield was 5.8 mg (71.5%). LC-MS (LR, ESI) = Calcd. for $C_{92}H_{107}N_{10}O_{10}S$: 1543.78924 (m/z), found: 1544.96 $[M+H]^+$. LC-MS (HR, ESI) = Calcd. for $C_{92}H_{107}N_{10}O_{10}S$: 1543.78924, found: 1544.7880 $[M+H]^+$.

Fmoc-d-Lys(Gd-DOTA)-Tyr-Gly-Gly-Phe-Met-NH_2 (12). Resin 7R (8 mg, ca. 10 % of the prior yield of 7R) was treated by the same procedure as for (4) above using Fmoc-d-Lys(Gd-DOTA)-NH_2 (5.3 mg, 5.82 μmol), followed by cleavage of the resultant resin also by the same procedure as above to yield of Fmoc-d-Lys(Gd-DOTA)-Tyr-Gly-Gly-Phe-Met-NH_2 (12). LC-MS (LR, ESI) = Calcd. for $C_{64}H_{81}GdN_{12}O_{16}S$: 1463.718 (m/z), found: 1464.51 $[M+H]^+$. LC-MS (HR, ESI) = Calcd. for $C_{64}H_{81}GdN_{12}O_{16}S$: 1463.48552, found: 1464.4897 $[M+H]^+$.

Fmoc-Leu-NH_2 (13). Xanthenyl linker resin (100.0 mg, 29.1 μmol) was pre-swelled in DMF (2 mL). After draining, mixture of 5% Piperazine, 2% DBU and 93% DMF (3 × 2 mL) were applied to the resin. DMF (3 × 2mL) was added to wash the beads. Fmoc-Leu-OH (20.6 mg, 58.2 μmol), was dissolved in 2 mL DMF, followed by addition of DIEA (49.7 μL, 29.1 μmol). HATU (21.0 mg, 55.3 μmol) was dissolved separately in 2 mL DMF added to the previous mixture and shaken. Then this solution was added to the resin within 1 minute. After being stirring for about 45 minutes at room temperature, the reaction solution was drained and washed with pure DMF (3 × 2 mL) and DCM (2 × 2 mL). A small amount of the resultant resin (8R) (10 μg), was removed and cleaved with 1% TFA in DCM (5 × 1 mL) for 1-2 min, drained through the vessel frit. The eluent was concentrated on a rotovap, dissolved in 0.5 mL methanol and assayed by LC-MS. LC-MS (LR, ESI) = Calcd. for $C_{21}H_{24}N_2O_3$: 352.428 (m/z), found: 353.25 $[M+H]^+$.

Fmoc-Phe-Leu-NH_2 (14). Resin 8R was treated by the same procedure as for (13) above using Fmoc-Phe-OH (22.5 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (9R) also by the same procedure as above to yield of Fmoc-Phe-Leu-NH_2 (14). LC-MS (LR, ESI) = Calcd. for $C_{30}H_{33}N_3O_4$: 499.602 (m/z), found: 500.32 $[M+H]^+$.

Fmoc-d-Lys-Phe-Leu-NH_2 (15). Resin 9R was treated by the same procedure as for (13) above using Fmoc-d-Lys(Mtt)-OH (36.4 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (10R) also by the same procedure as above to yield of Fmoc-d-Lys-Phe-Leu-NH_2 (15). LC-MS (LR, ESI) = Calcd. for $C_{36}H_{45}N_5O_5$: 627.774 (m/z), found: 628.43 $[M+H]^+$.

Fmoc-Gln-d-Lys-Phe-Leu-NH_2 (16). Resin 10R was treated by the same procedure as for (13) above using Fmoc-Gln-OH (22.5 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (11R) also by the same procedure as above to yield of Fmoc-Gln-d-Lys-Phe-Leu-NH_2 (16). LC-MS (LR, ESI) = Calcd. for $C_{41}H_{53}N_7O_7$: 755.904 (m/z), found: 756.47 $[M+H]^+$.

Fmoc-Tyr-Gln-d-Lys-Phe-Leu-NH_2 (17). Resin 11R was treated by the same procedure as for (13) above using Fmoc-Tyr(tBu)-OH (26.7 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (12R) also by the same procedure as above to yield of Fmoc-Tyr-Gln-d-Lys-Phe-Leu-NH_2 (17). LC-MS (LR, ESI) = Calcd. for $C_{50}H_{62}N_8O_9$: 919.077 (m/z), found: 919.56 $[M+H]^+$.

Fmoc-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (18). Resin 12R was treated by the same procedure as for (13) above using Fmoc-Ala-OH (18.1 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (13R) also by the same procedure as above to yield of Fmoc-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (18). LC-MS (LR, ESI) = Calcd. for $C_{57}H_{75}N_9O_{10}$: 1045.56369 (m/z), found: 1046.64 $[M+H]^+$.

Fmoc-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (19). Resin 13R was treated by the same procedure as for (13) above using Fmoc-Ala-OH (18.1 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (14R) also by the same procedure as above to yield of Fmoc-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (19). LC-MS (LR, ESI) = Calcd. for $C_{60}H_{80}N_{10}O_{11}$: 1116.60080 (m/z), found: 1117.68 $[M+H]^+$.

Fmoc-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (20). Resin 14R was treated by the same procedure as for (13) above using Fmoc-Glu(OtBu)-OH (24.8 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (15R) also by the same procedure as above to yield of Fmoc-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (20). LC-MS (LR, ESI) = Calcd. for $C_{69}H_{95}N_{11}O_{14}$: 1301.70600 (m/z), found: 1302.82 $[M+H]^+$.

Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (21). Resin 15R was treated by the same procedure as for (13) above using Fmoc-d-Nle-OH (20.6 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (16R) also by the same procedure as above to yield of Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (21). LC-MS (LR, ESI) = Calcd. for $C_{75}H_{106}N_{12}O_{15}$: 1414.79006 (m/z), found: 1415.91 $[M+H]^+$.

Fmoc-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (22). Resin 16R was treated by the same procedure as for (13) above using Fmoc-Trp(Boc)-OH (30.6 mg, 58.2 μmol), followed by cleavage of a small amount the resultant resin (17R) also by the same procedure as above to yield of Fmoc-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (22). LC-MS (LR, ESI) = Calcd. for $C_{91}H_{124}N_{14}O_{18}$: 1702.044 (m/z), found: 1703.19 $[M+H]^+$. LC-MS (HR, ESI) = Calcd. for $C_{91}H_{124}N_{14}O_{18}$: 1700.92180 (m/z), found: 1701.9264 $[M+H]^+$.

Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (23). Take a small amount of resin 17R (10 μg), treated by the same procedure as for (13) above using Fmoc-d-Lys(Cy5.5)-NH_2 (5.43 mg, 5.82 μmol), followed by cleavage of the resultant resin (18R) also by the same procedure as above to yield of Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (23). LC-MS (LR, ESI) = Calcd. for $C_{137}H_{177}N_{18}O_{20}$: 2395.985 (m/z), found: 1198.32 $[M+2H]^+/2$. LC-MS (HR, ESI) = Calcd. for $C_{137}H_{177}N_{18}O_{20}$: 2394.33866 (m/z), found: 1198.1732 $[M+2H]^+/2$.

H-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2 (24). Take a small amount of resin 18R (10 μg), treated with Fmoc deprotection method, followed by cleavage of the resultant resin also by the same procedure as above to yield of NH_2-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2 (24). LC-MS (LR, ESI) = Calcd. for $C_{109}H_{143}N_{18}O_{16}$: 1960.08800 (m/z), found: 981.28 $[M+2H]^+/2$. LC-MS (HR, ESI) = Calcd. for $C_{109}H_{143}N_{18}O_{16}$: 1960.08800 (m/z), found: 981.05194 $[M+2H]^+/2$.

Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (25). Take a small amount of resin 17R (10 μg), treated by the same procedure as for (13) above using Fmoc-d-Lys(Gd-DOTA)-NH_2 (5.3 mg, 5.82 μmol), followed by cleavage of the resultant resin also by the same procedure as above to yield of Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 (24). LC-MS (LR, ESI) = Calcd. for $C_{113}H_{159}GdN_{20}O_{26}$: 2370.846 (m/z), found:

1186.64 $[M+2H]^+/2$. LC-MS (HR, ESI) = Calcd. for $C_{113}H_{159}GdN_{20}O_{26}$: 2370.09754 (m/z), found: 1186.0563 $[M+2H]^+/2$.

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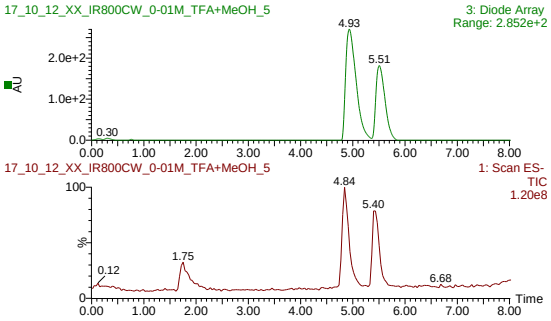
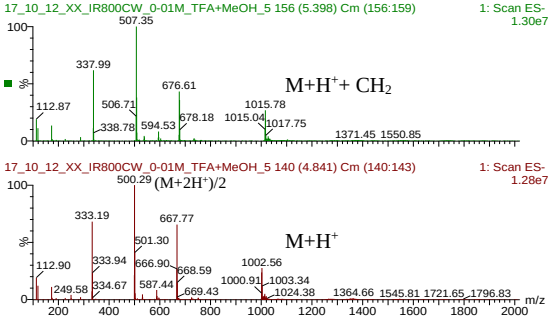
Appendix I: Analytical Data

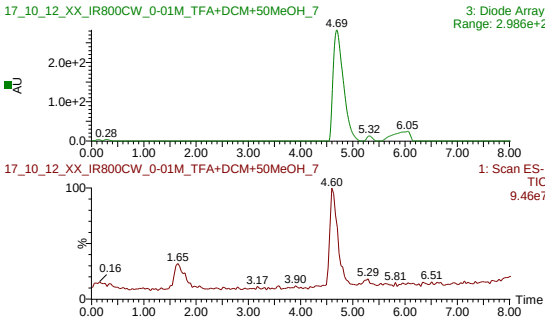
Stability test of IR800

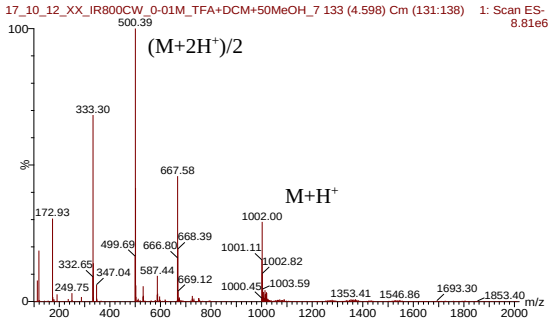




LC-MS total diode array chromatogram (TDA)
Peak 5.51 is Methyl adducts with no DCM present

Mass spectra of parent dye and methyl adduct (top)

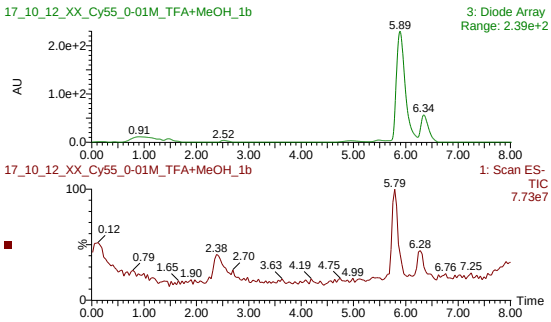


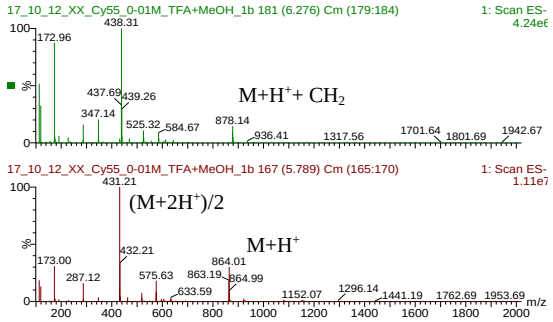


LC-MS total diode array chromatogram (TDA)
No methyl adducts when DCM is present

Mass spectra of parent dye

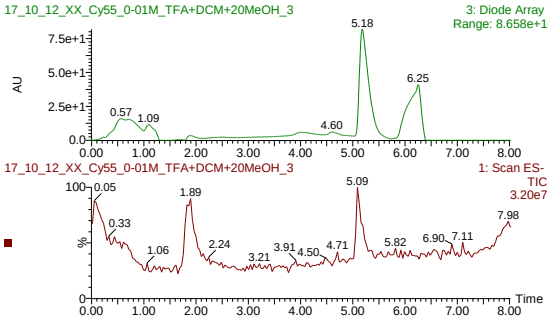
Stability test of Cy5.5



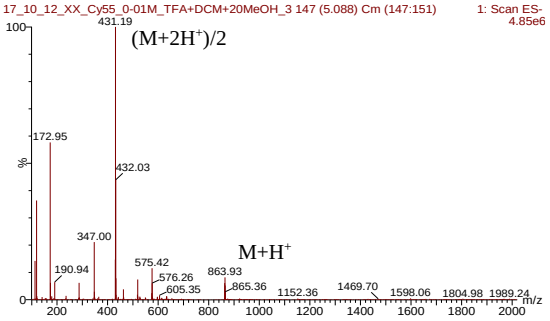


LC-MS total diode array chromatogram (TDA)
Peak 6.34 is Methyl adducts with no DCM present

Mass spectra of parent dye and methyl adduct (top)

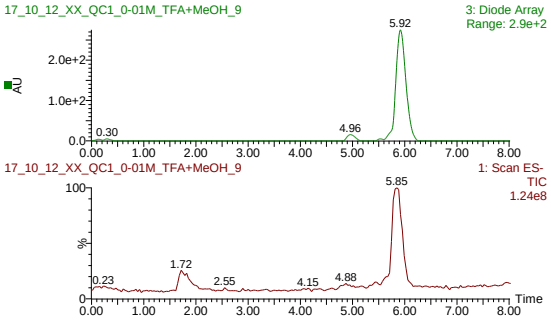


LC-MS total diode array chromatogram (TDA)
No methyl adducts when DCM is present

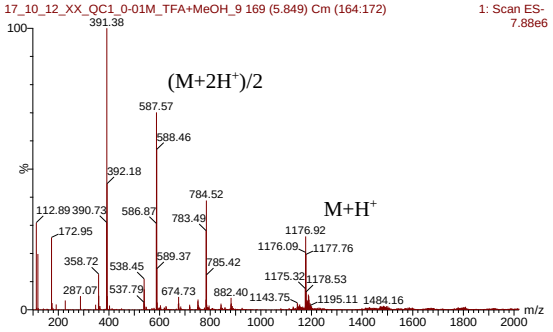


Mass spectra of parent dye

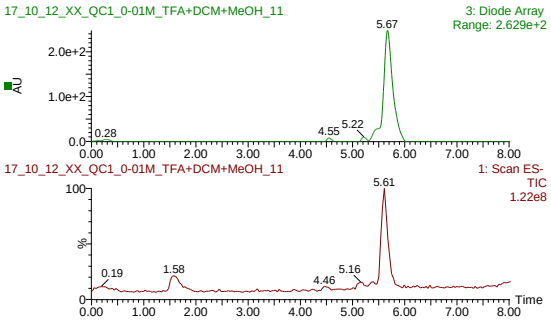
Stability test of QC1



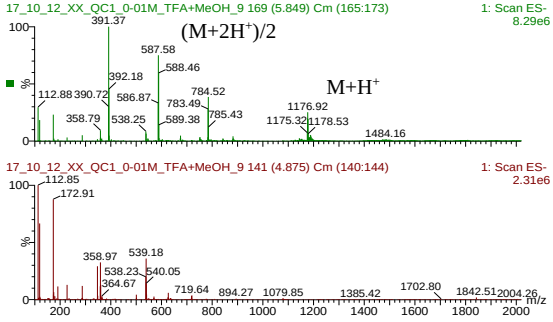
LC-MS total diode array chromatogram (TDA)



Mass spectra of parent dye

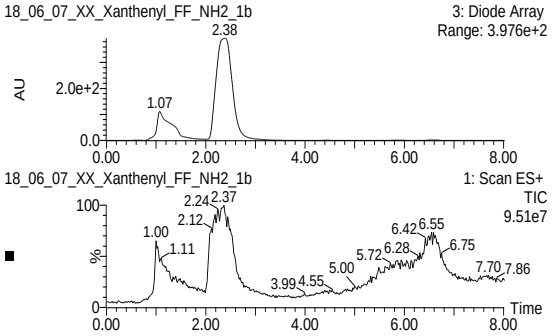


LC-MS total diode array chromatogram (TDA)

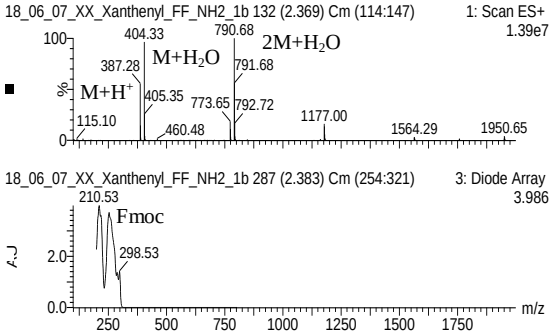


Mass spectra of parent dye

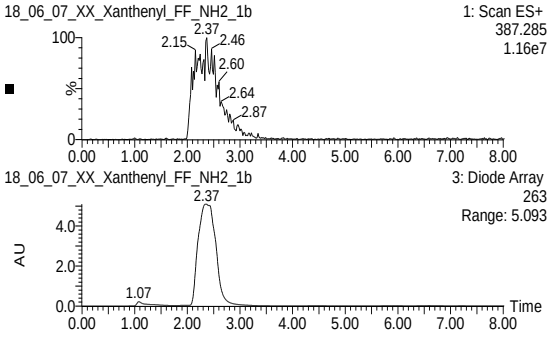
Fmoc-Phe-NH_2 **1**



LC-MS Total Diode Array Chromatogram (TDA)
Total Ion Chromatogram (TIC)

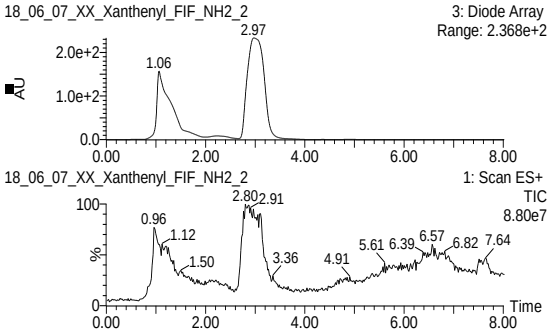


Mass Spectra and UV Spectra of cleaved product

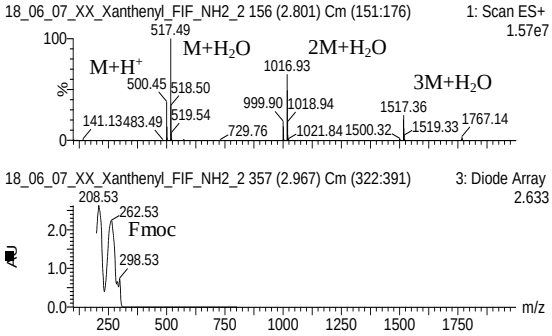


Extracted Ion Chromatogram (XIC) at 387 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

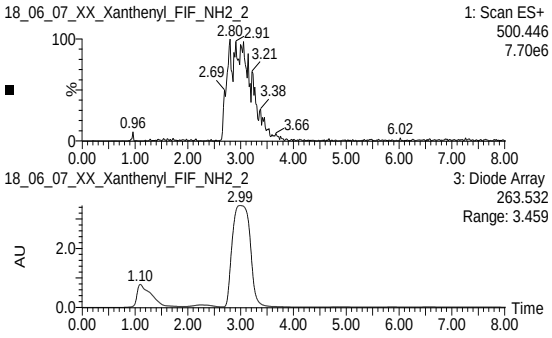
Fmoc-Ile-Phe-NH_2 **2**



LC-MS Total Diode Array Chromatogram (TDA)
Total Ion Chromatogram (TIC)

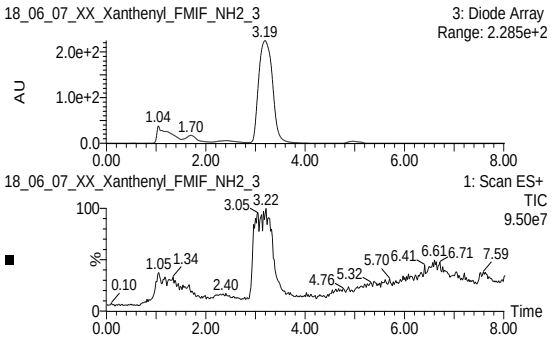


Mass Spectra and UV Spectra of cleaved product

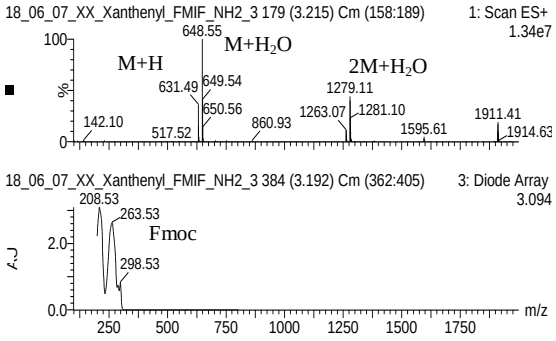


Extracted Ion Chromatogram (XIC) at 500 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

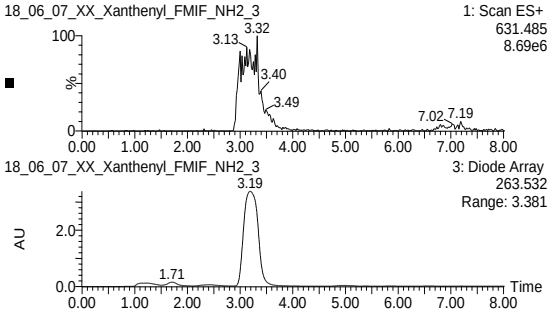
Fmoc-Met-Ile-Phe-NH_2 **3**



LC-MS Total Diode Array Chromatogram (TDA)
Total Ion Chromatogram (TIC)

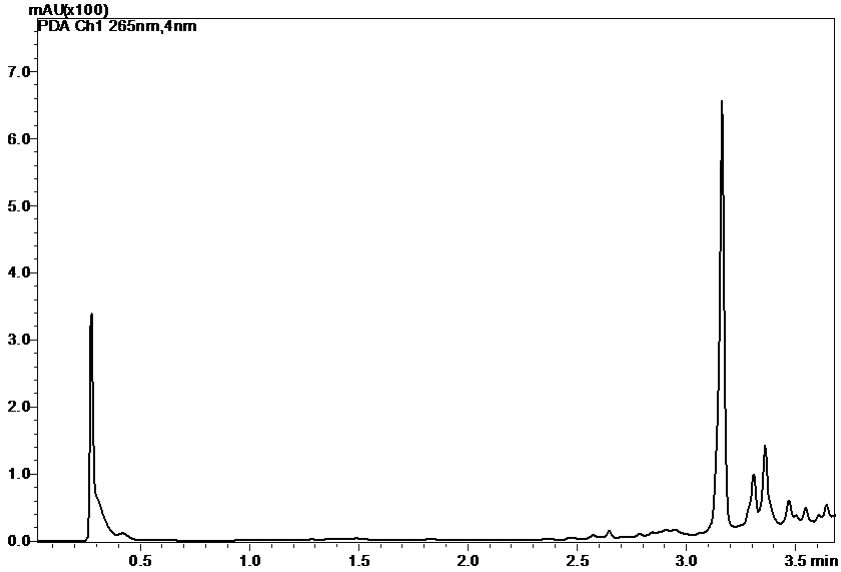


Mass Spectra and UV Spectra of cleaved product

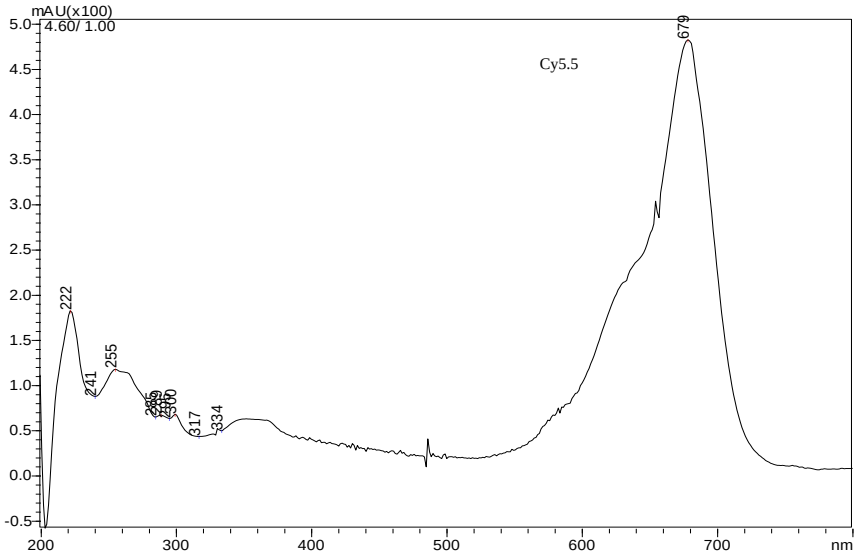


Extracted Ion Chromatogram (XIC) at 631 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

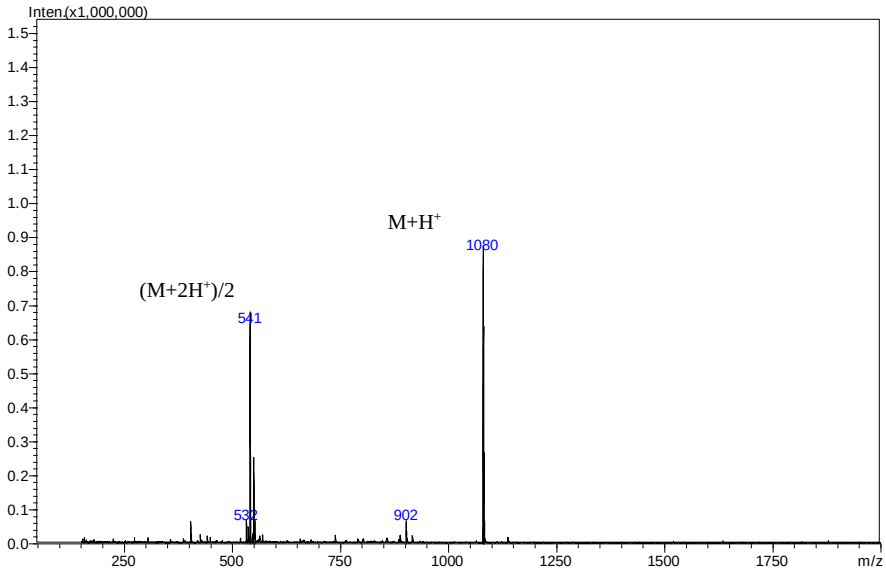
Fmoc-d-Lys(Cy5.5)-Phe-NH_2 **4**



Single wavelength chromatogram (SWC) at 265 nm

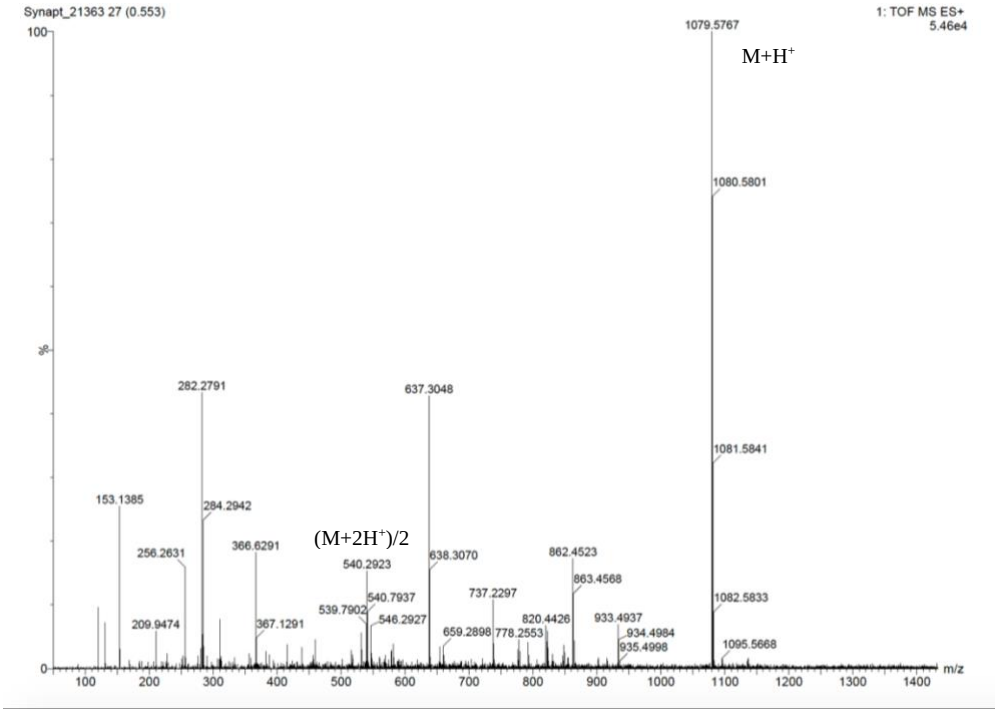


UV-Vis-NIR spectra shows Cy5.5 dye is present in product

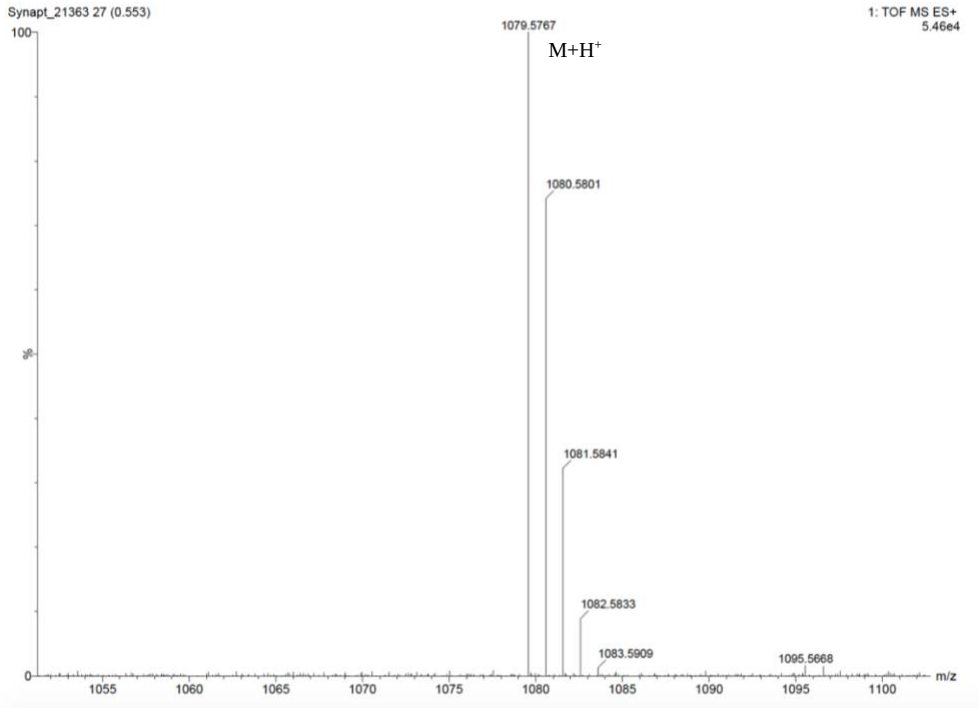


Mass spectra of product

HRMS of Fmoc-d-Lys(Cy5.5)-Phe-NH_2

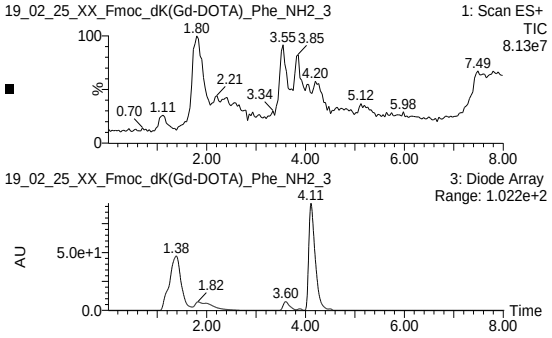


High resolution mass spectra of product

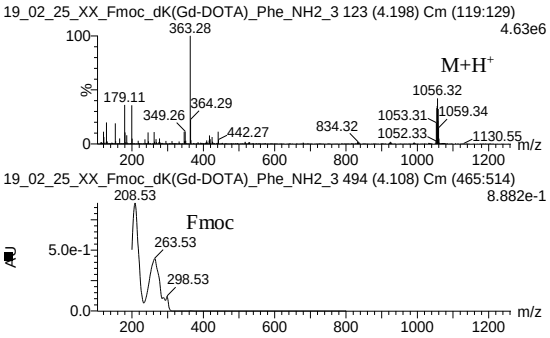


Expanded high resolution mass spectra of product

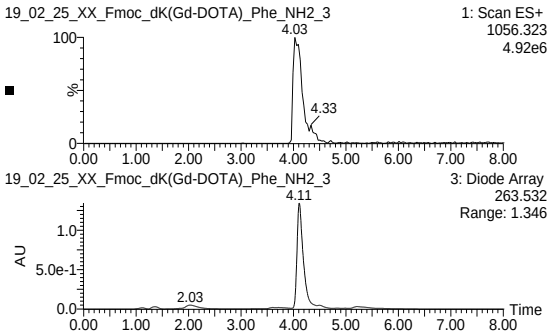
Fmoc-d-Lys(Gd-DOTA)-Phe-NH_2 **5**



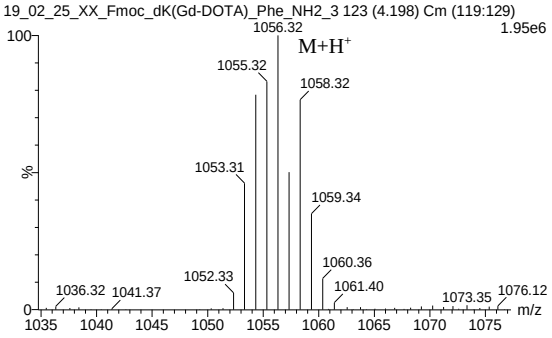
Total Ion Chromatogram (TIC)
LC-MS Total Diode Array Chromatogram
product at 4.11 min



Mass Spectra and UV Spectra of cleaved product

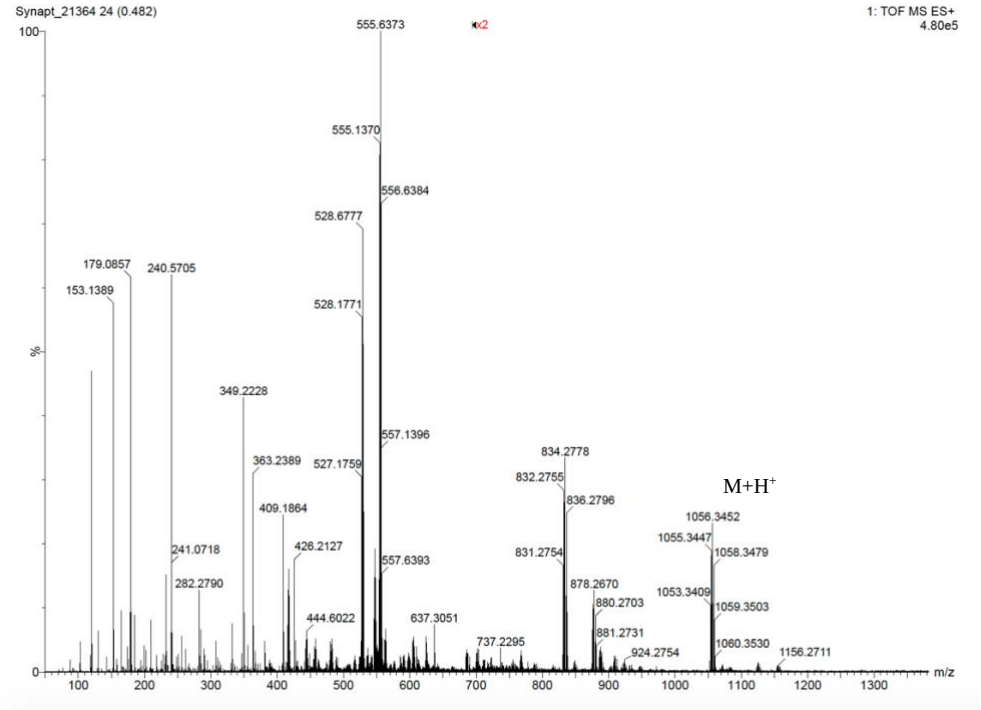


Extracted Ion Chromatogram (XIC) at 1056 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

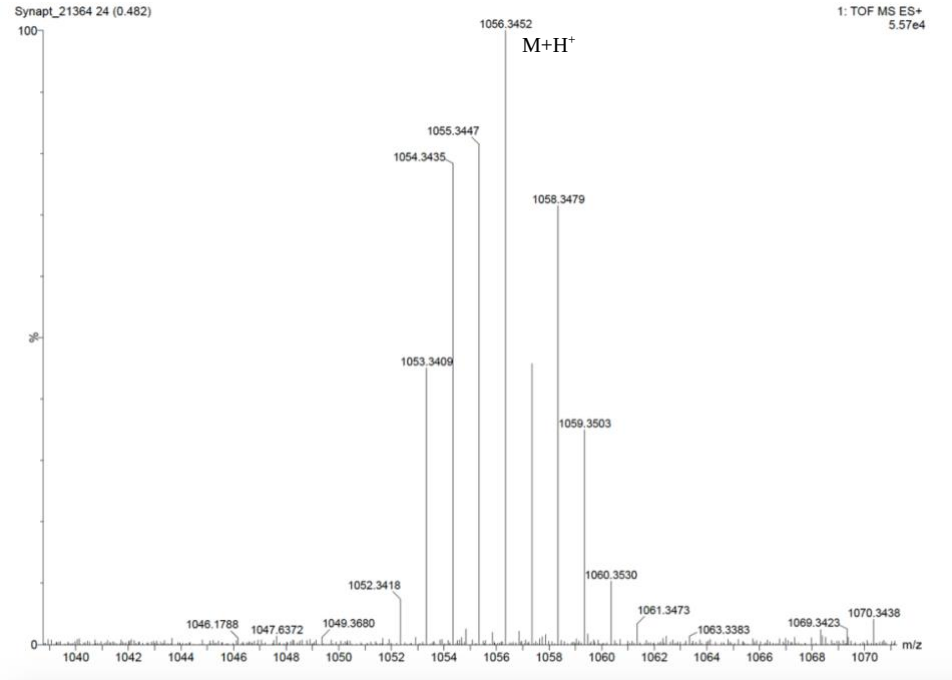


Expanded mass spectra of product showing Gd isotope pattern

HRMS of Fmoc-d-Lys(Gd-DOTA)-Phe-NH_2

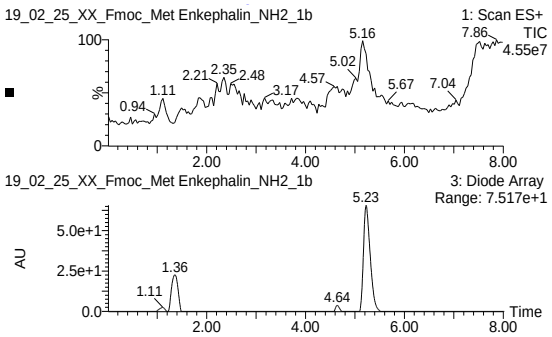


High resolution mass spectra of product

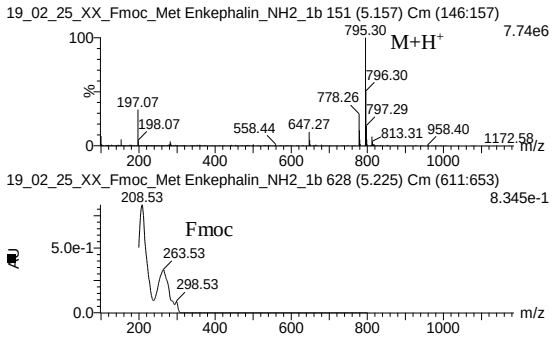


Expanded high resolution mass spectra of Gd isotope pattern

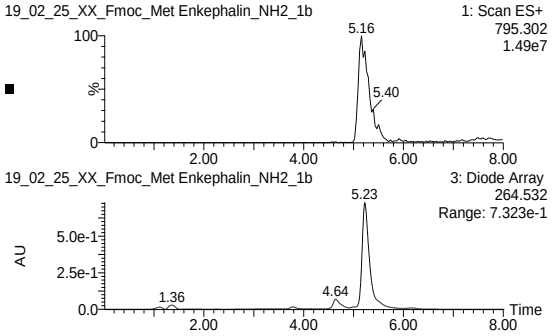
Fmoc-Tyr-Gly-Gly-Phe-Met-NH_2 **10**



Total Ion Chromatogram (TIC)
LC-MS Total Diode Array Chromatogram
product at 5.23 min

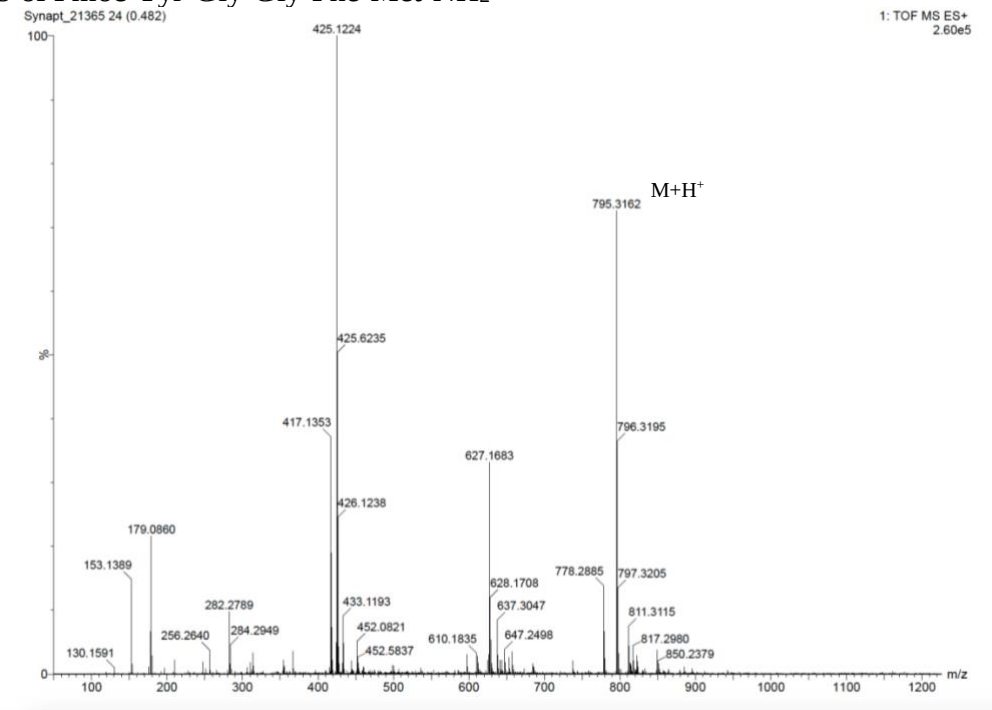


Mass Spectra and UV Spectra of cleaved product



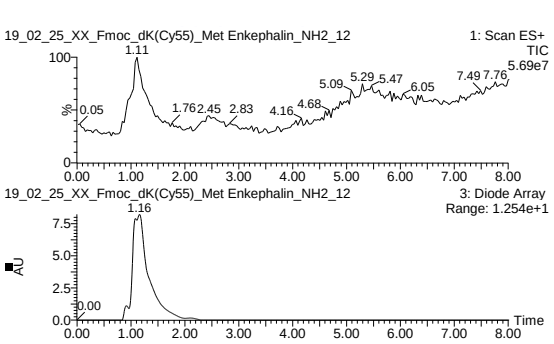
Extracted Ion Chromatogram (XIC) at 795 amu.
Single Wavelength Chromatogram (SWC) at 264 nm

HRMS of Fmoc-Tyr-Gly-Gly-Phe-Met-NH_2

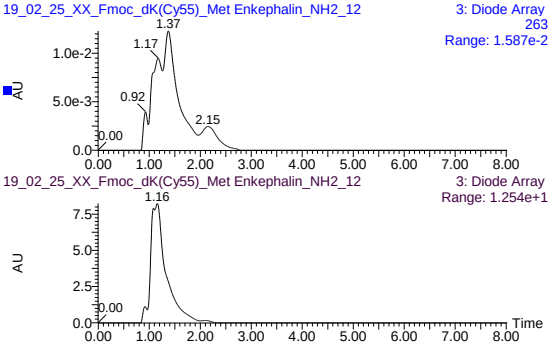


High resolution mass spectra of product

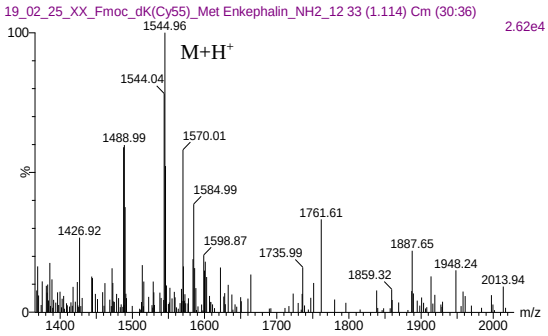
Fmoc-d-Lys(Cy5.5)-Tyr-Gly-Gly-Phe-Met-NH_2 **11**

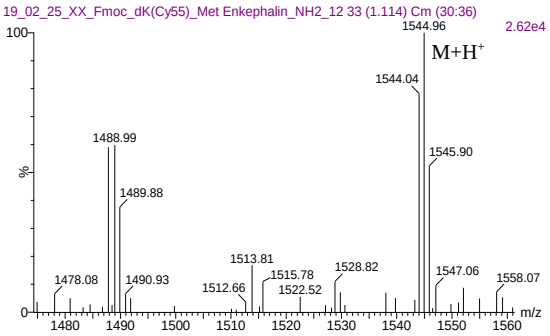


LC-MS Total Diode Array Chromatogram (TDA)
Total Ion Chromatogram (TIC)



Single wavelength chromatogram at 263 nm

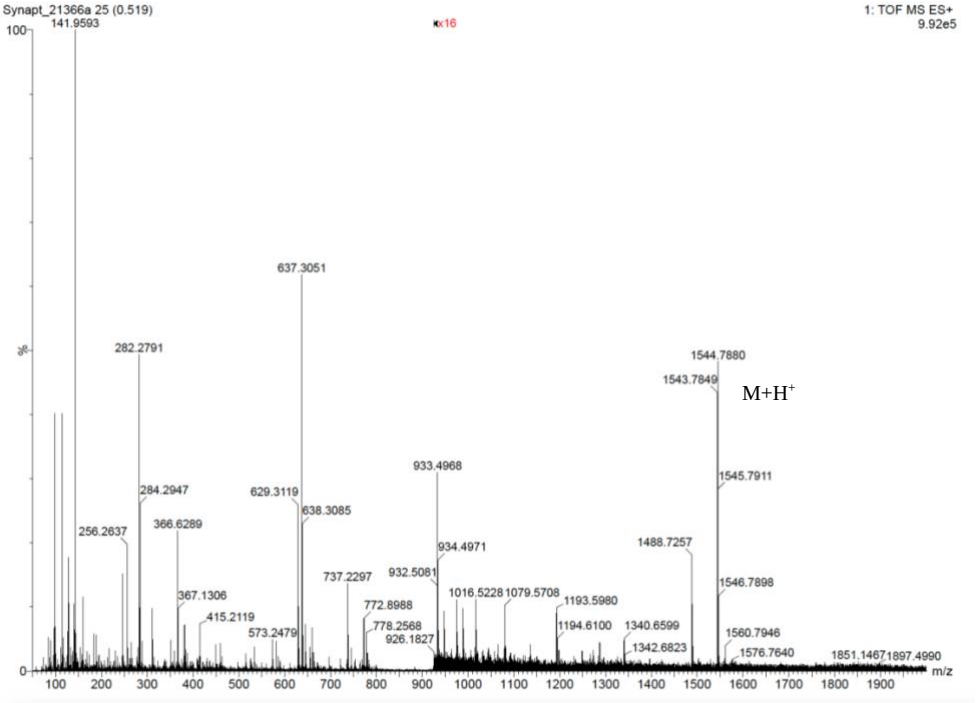




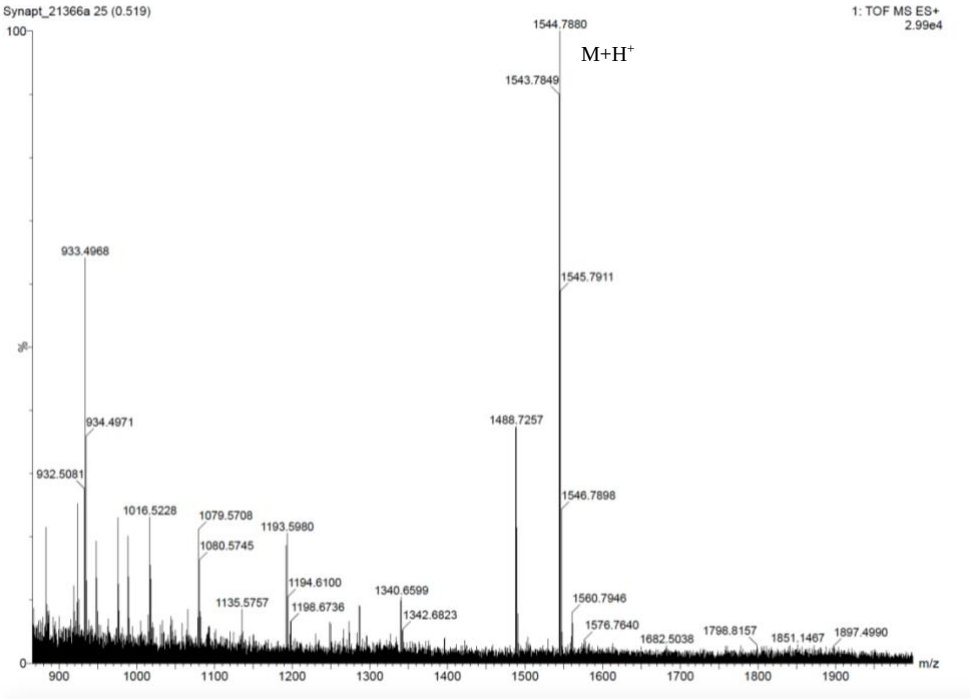
Expanded mass spectra of product 1488 amu.
(mass of product with protected groups 1544 amu.)

Expanded mass spectra

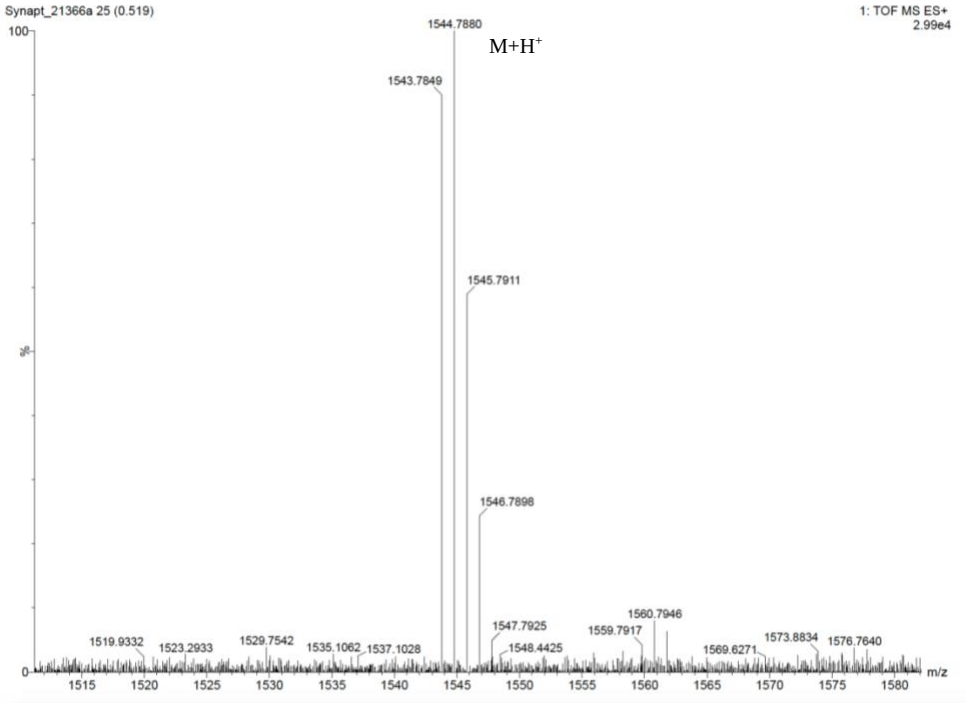
HRMS of Fmoc-d-Lys(Cy5.5)-Met enkephalin-NH_2



High resolution mass spectra of product

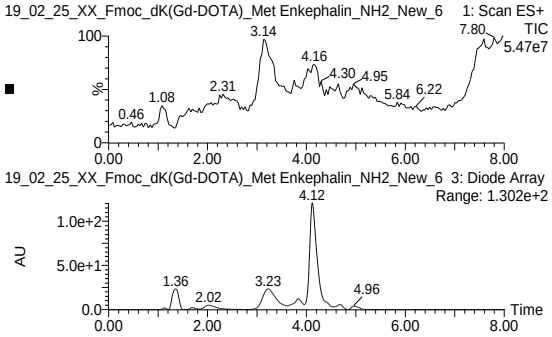


Expanded High resolution mass spectra of product

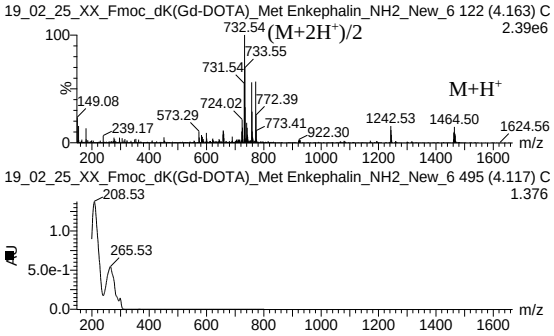


Expanded High resolution mass spectra of product (with protecting groups)

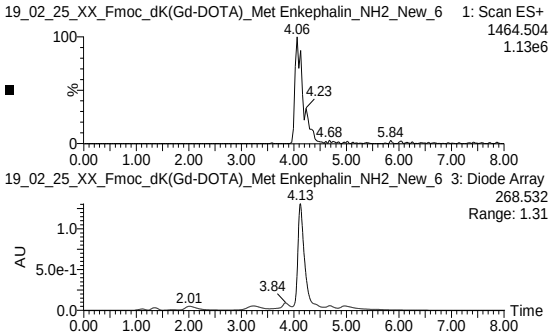
Fmoc-d-Lys(Gd-DOTA)-Tyr-Gly-Gly-Phe-Met-NH_2 **12**



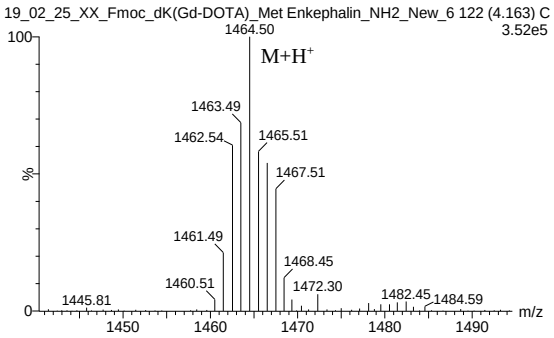
Total Ion Chromatogram (TIC)
LC-MS Total Diode Array Chromatogram
product at 4.12 min



Mass Spectra and UV Spectra of cleaved product

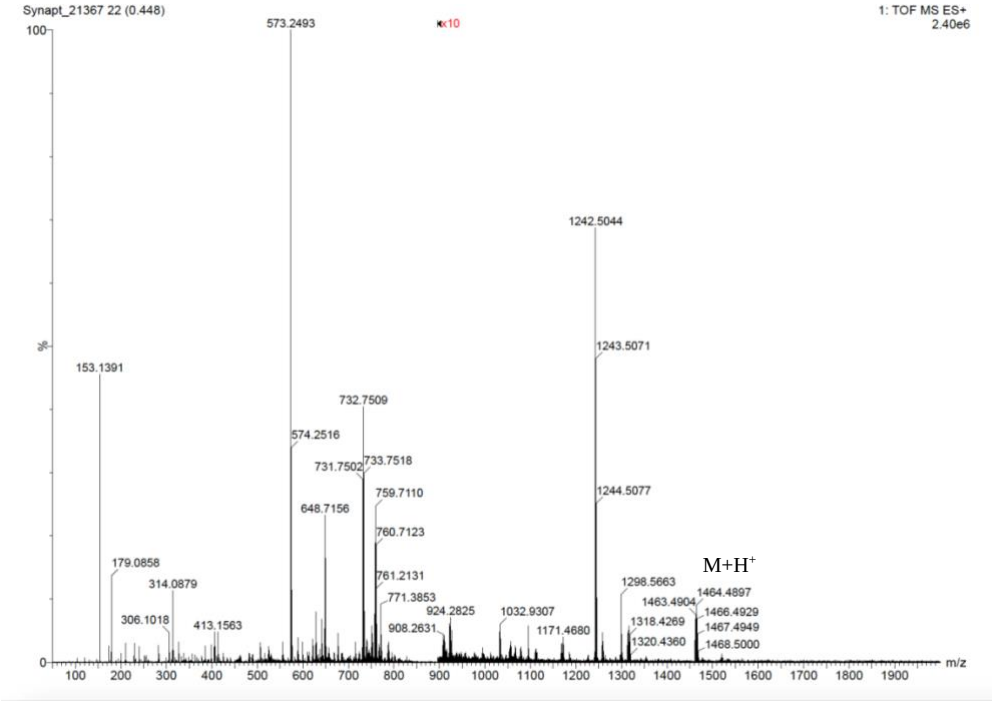


Extracted Ion Chromatogram (XIC) at 1464 amu.
Single Wavelength Chromatogram (SWC) at 268 nm

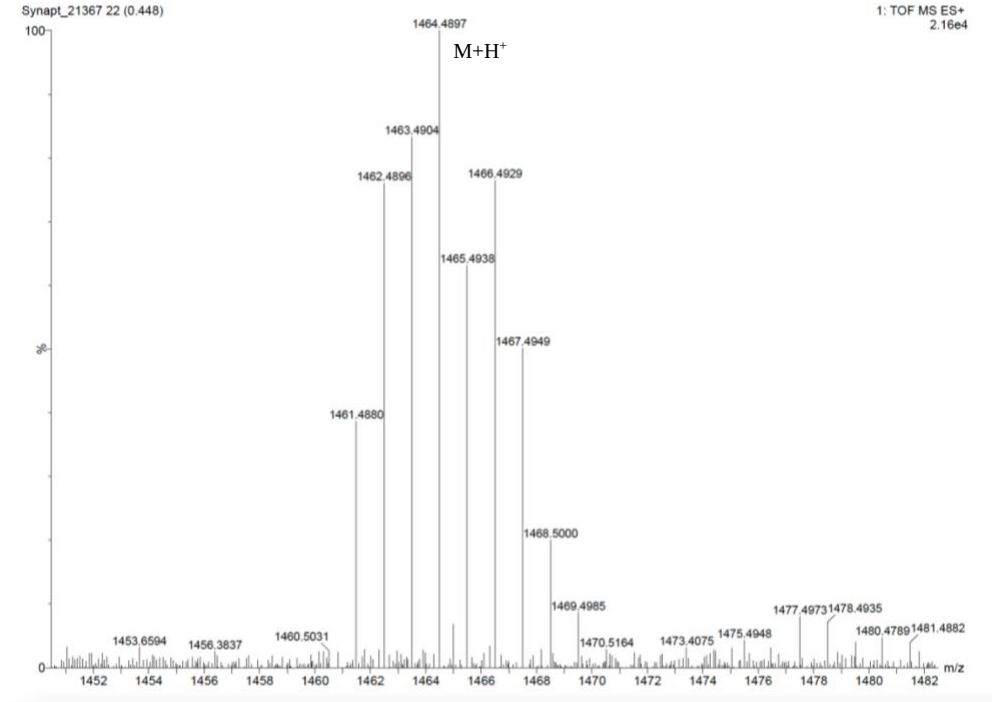


Expanded mass spectra of product showing
Gd isotope pattern

HRMS of Fmoc-d-Lys(Gd-DOTA)-Met enkephalin-NH_2

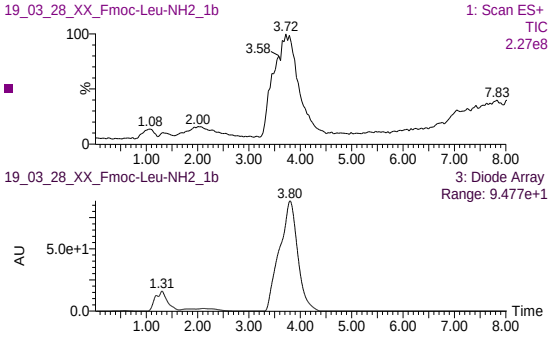


High resolution mass spectra of product (with protecting groups)

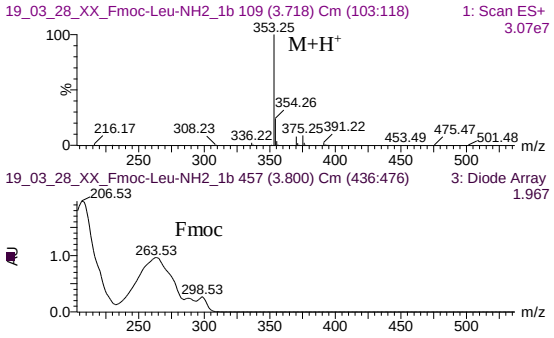


Expanded High resolution mass spectra of product (with protecting groups)

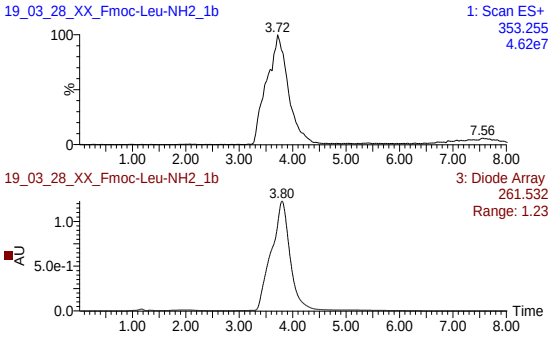
Fmoc-Leu-NH_2 **13**
(First amino acid in SPPS of 18-4)



LC-MS Total Diode Array Chromatogram (TDA)
Total Ion Chromatogram (TIC)

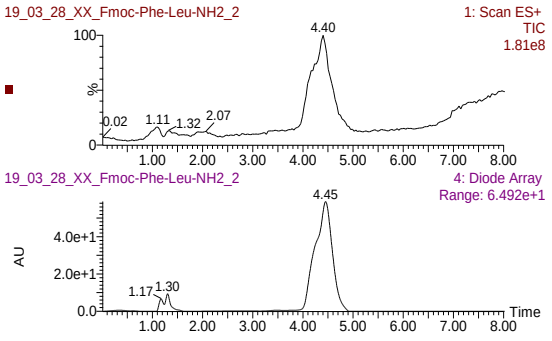


Mass Spectra and UV Spectra of cleaved product

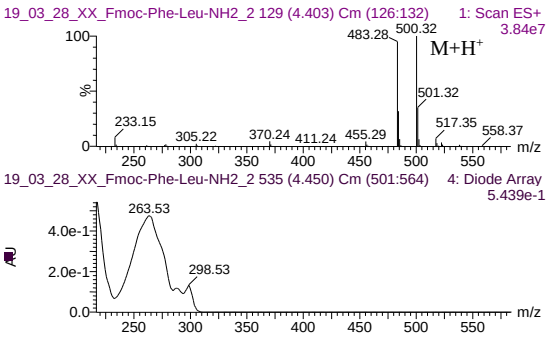


Extracted Ion Chromatogram (XIC) at 353 amu.
Single Wavelength Chromatogram (SWC) at 261 nm

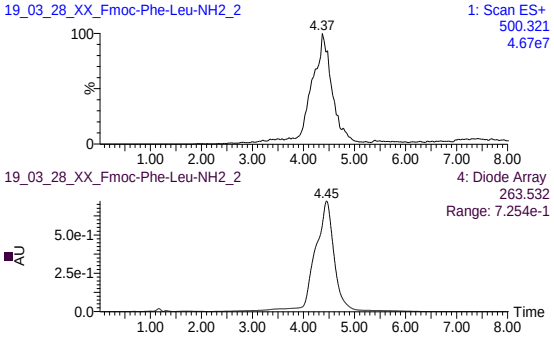
Fmoc-Phe-Leu-NH_2 **14**
(Di-peptide in SPPS of 18-4)



TIC & TDA of di-peptide, product at 4.45 min
(peak is broad due to lipophilicity)

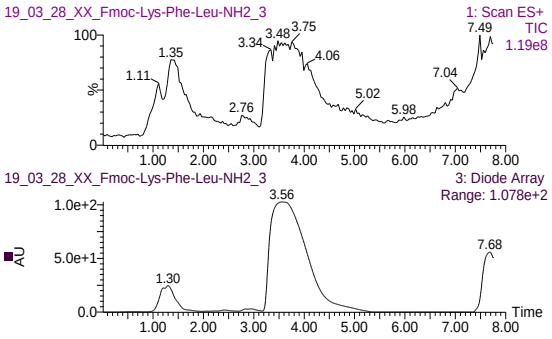


Mass Spectra and UV Spectra of cleaved product

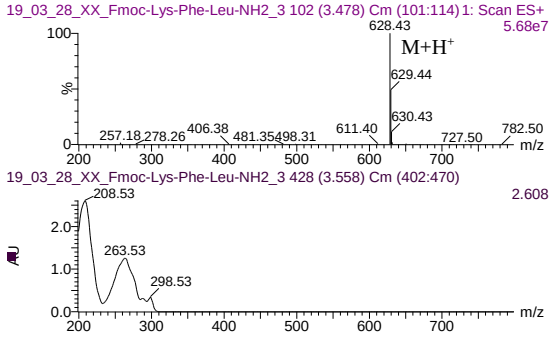


Extracted Ion Chromatogram (XIC) at 500 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

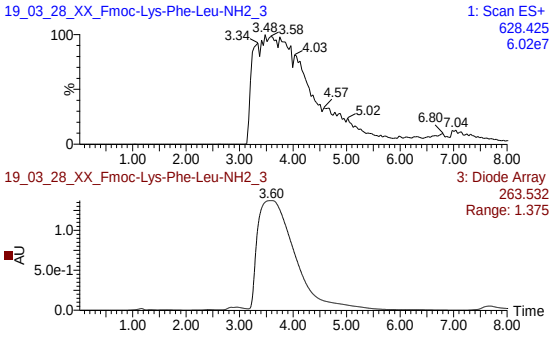
Fmoc-d-Lys-Phe-Leu-NH_2 **15**
(Tri-peptide in SPPS of 18-4)



TIC & TDA of tri-peptide, product at 3.56 min
(peak is broad due to lipophilicity)

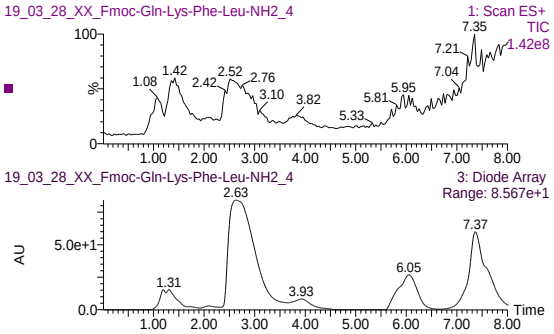


Mass Spectra and UV Spectra of cleaved product

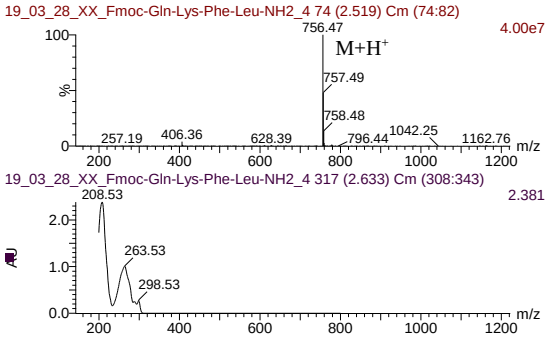


Extracted Ion Chromatogram (XIC) at 628 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

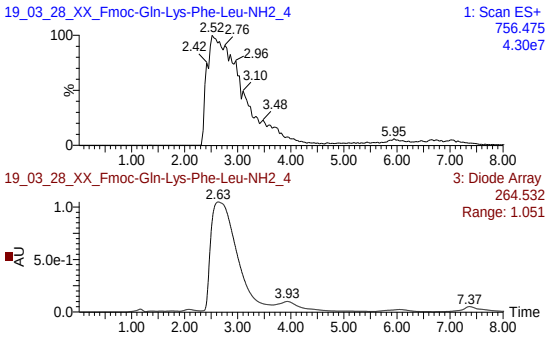
Fmoc-Gln-d-Lys-Phe-Leu-NH_2 **16**
(Tetra-peptide in SPPS of 18-4)



TIC & TDA of tetra-peptide, product at 2.63 min (peak is broad due to lipophilicity)

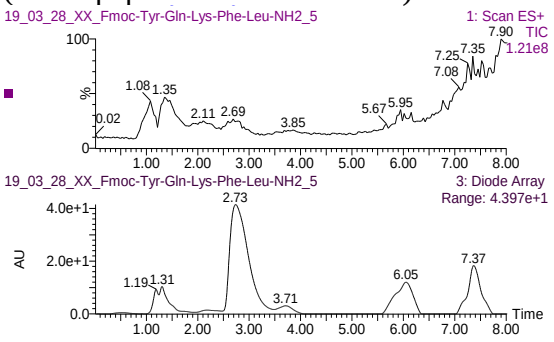


Mass Spectra and UV Spectra of cleaved product

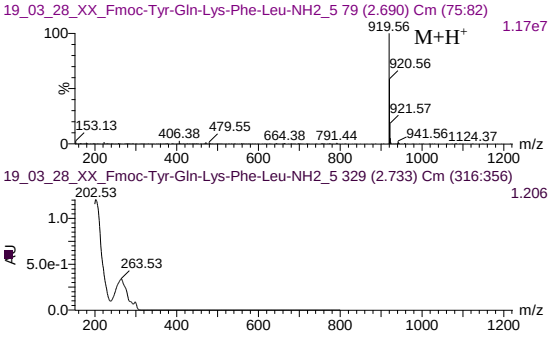


Extracted Ion Chromatogram (XIC) at 756 amu.
Single Wavelength Chromatogram (SWC) at 264 nm

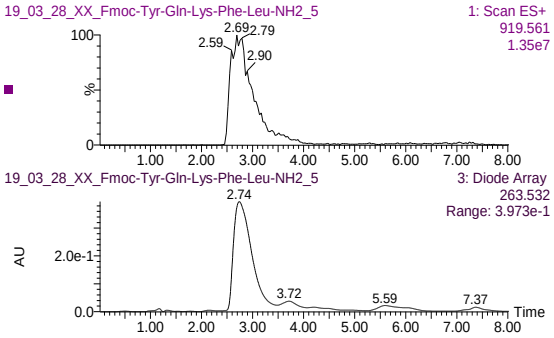
Fmoc-Tyr-Gln-d-Lys-Phe-Leu-NH_2 **17**
(Penta-peptide in SPPS of 18-4)



TIC & TDA of penta-peptide, product at 2.73 min (peak is broad due to lipophilicity)

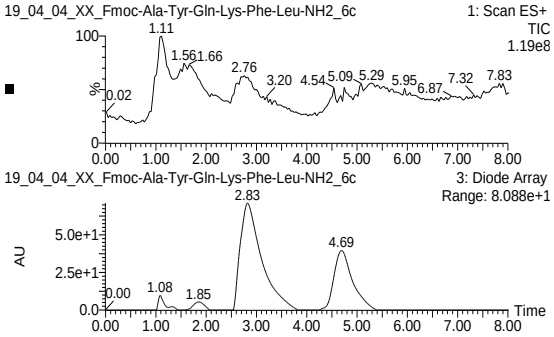


Mass Spectra and UV Spectra of cleaved product

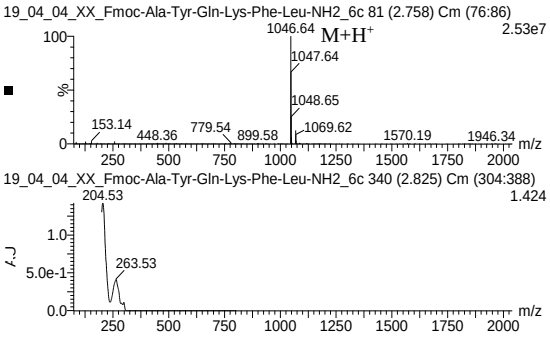


Extracted Ion Chromatogram (XIC) at 919 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

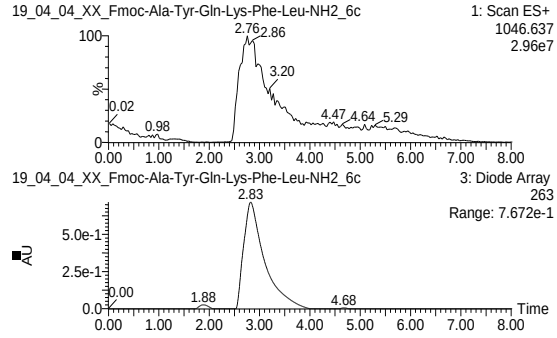
Fmoc-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **18**
(Hexa-peptide in SPPS of 18-4)



TIC & TDA of hexa-peptide, product at 2.83 min
(peak is broad due to lipophilicity)

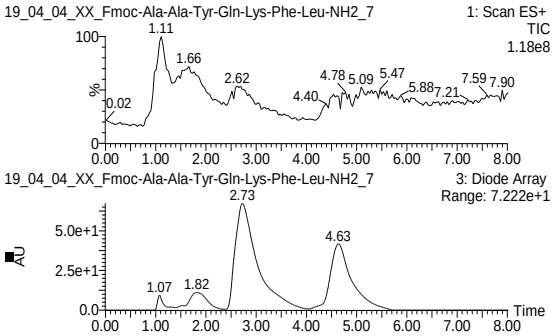


Mass Spectra and UV Spectra of cleaved product

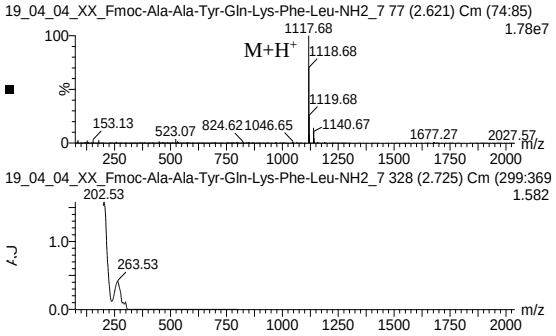


Extracted Ion Chromatogram (XIC) at 1046 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

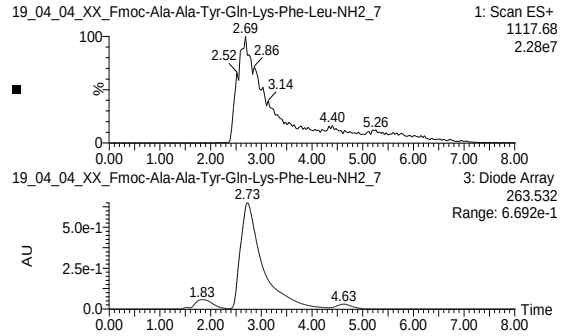
Fmoc-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **19**
(Hepta-peptide in SPPS of 18-4)



TIC & TDA of hepta-peptide, product at 2.73 min (peak is broad due to lipophilicity)

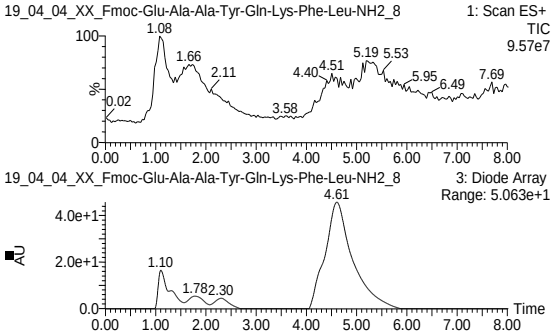


Mass Spectra and UV Spectra of cleaved product

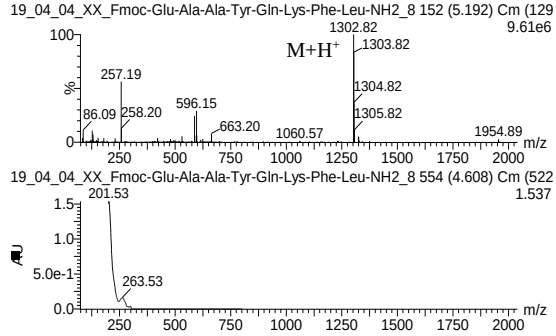


Extracted Ion Chromatogram (XIC) at 1117 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

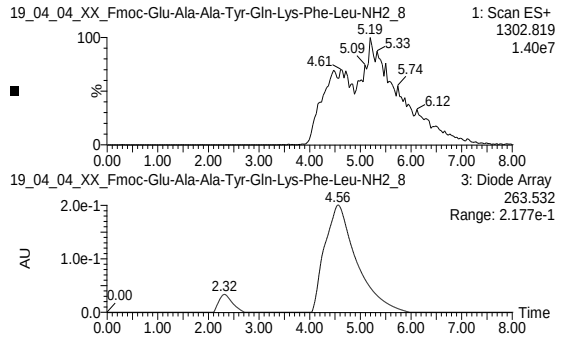
Fmoc-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **20**
(Octa-peptide in SPPS of 18-4)



TIC & TDA of octa-peptide, product at 4.61 min (peak is broad due to lipophilicity)

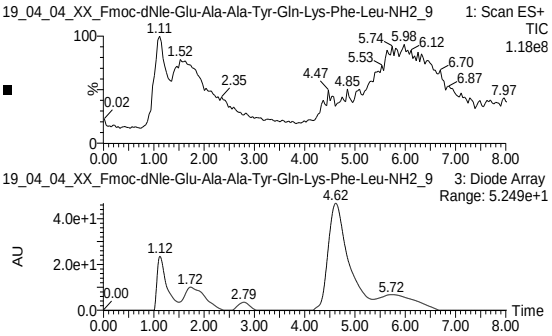


Mass Spectra and UV Spectra of cleaved product

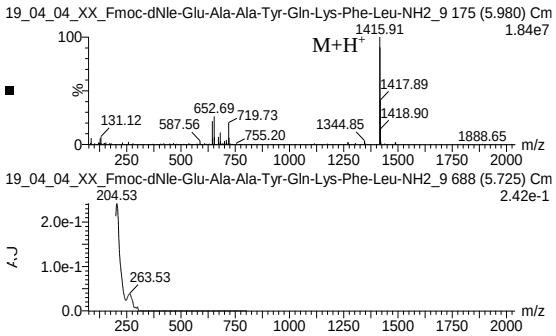


Extracted Ion Chromatogram (XIC) at 1302 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

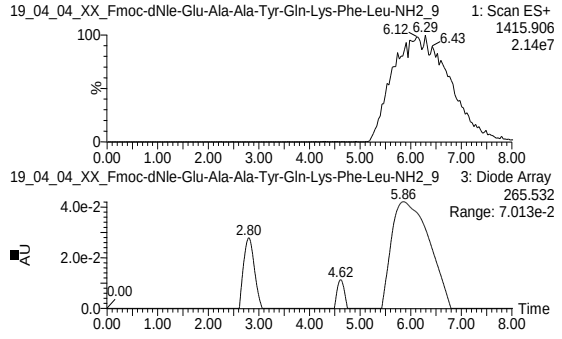
Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **21**
(Nona-peptide in SPPS of 18-4)



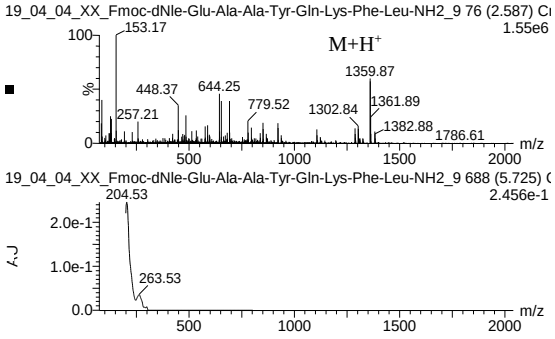
TIC & TDA of nona-peptide, product at 5.72 min
(peak is broad due to lipophilicity)



Mass Spectra and UV Spectra of cleaved product
(With two t-butyl protecting groups)

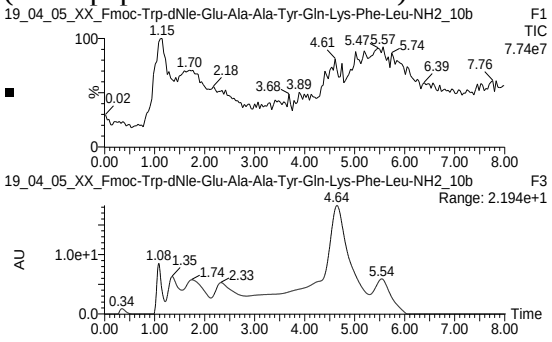


Extracted Ion Chromatogram (XIC) at 1415 amu.
Single Wavelength Chromatogram (SWC) at 265 nm

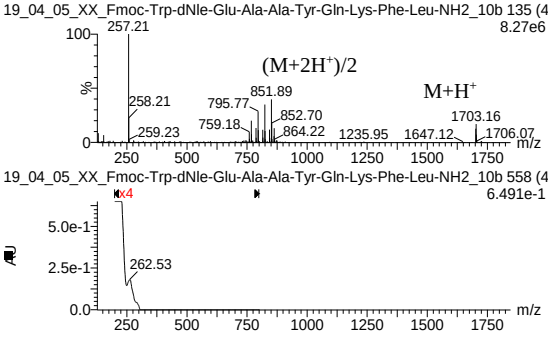


Mass Spectra and UV Spectra of cleaved product
(With one t-butyl protecting groups, 1359.87 amu.)
(Without protecting groups, 1302.84 amu.)

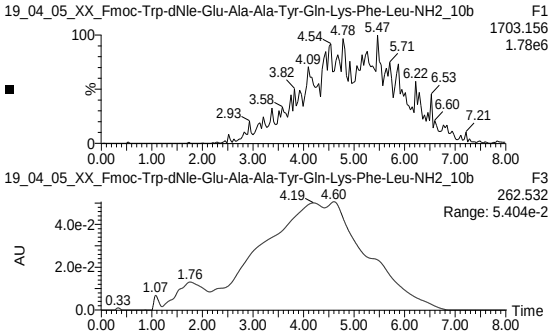
Fmoc-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **22**
(Deca-peptide in SPPS of 18-4)



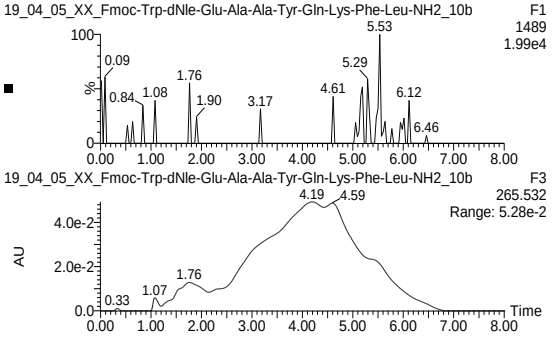
TIC & TDA of deca-peptide, product at 3.74 min (peak is broad due to lipophilicity)



Mass Spectra and UV Spectra of cleaved product

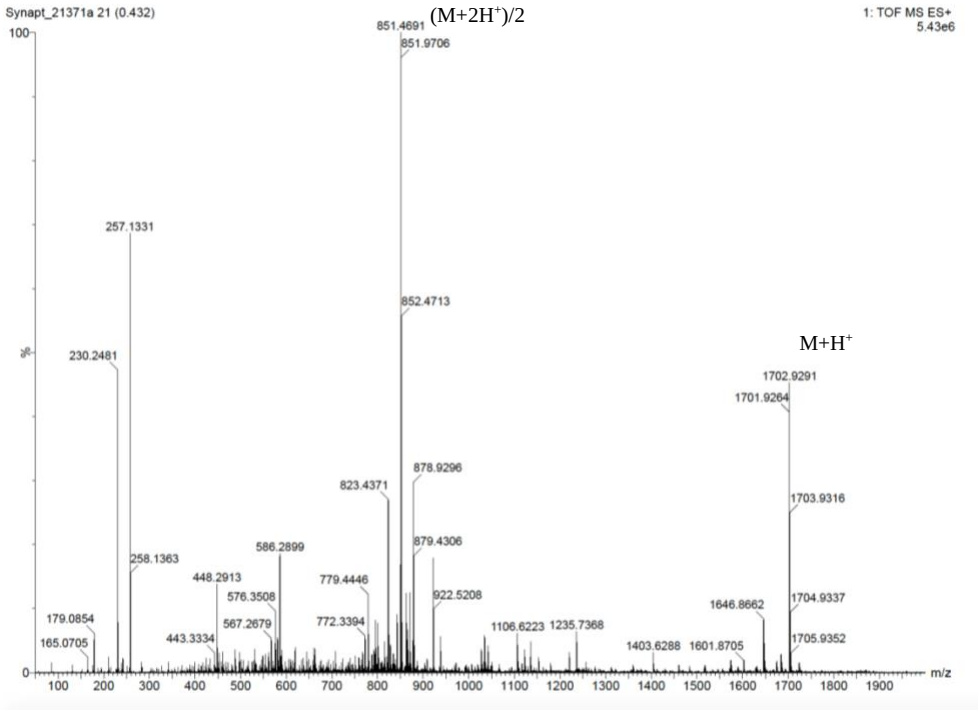


Extracted Ion Chromatogram (XIC) at 1703 amu.
Single Wavelength Chromatogram (SWC) at 265 nm

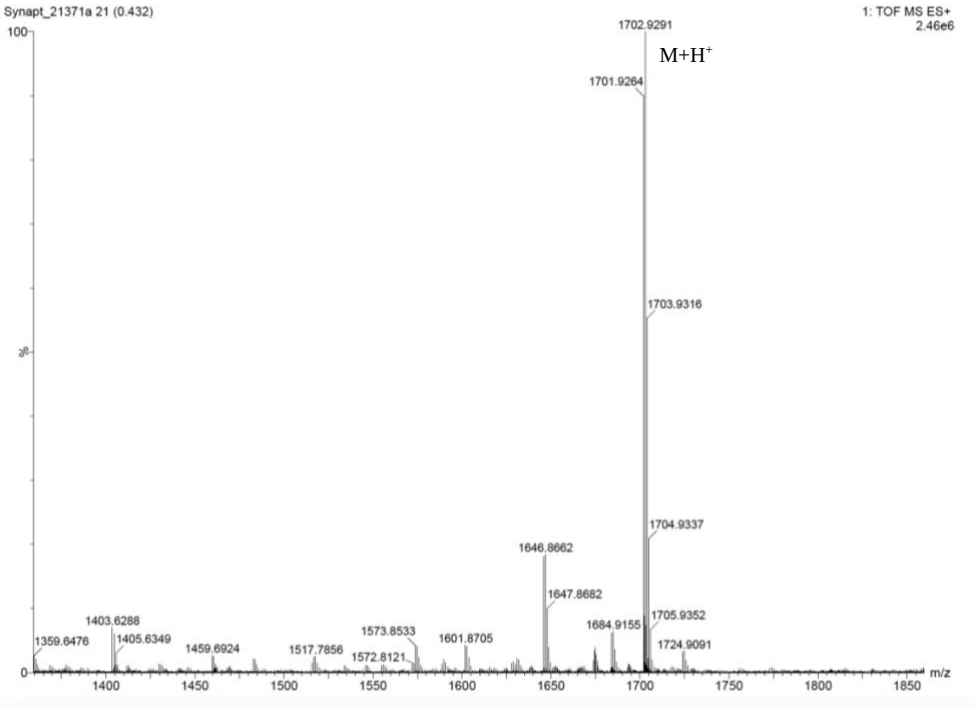


Extracted Ion Chromatogram (XIC) at 1489 amu.
(Product with no protecting groups)

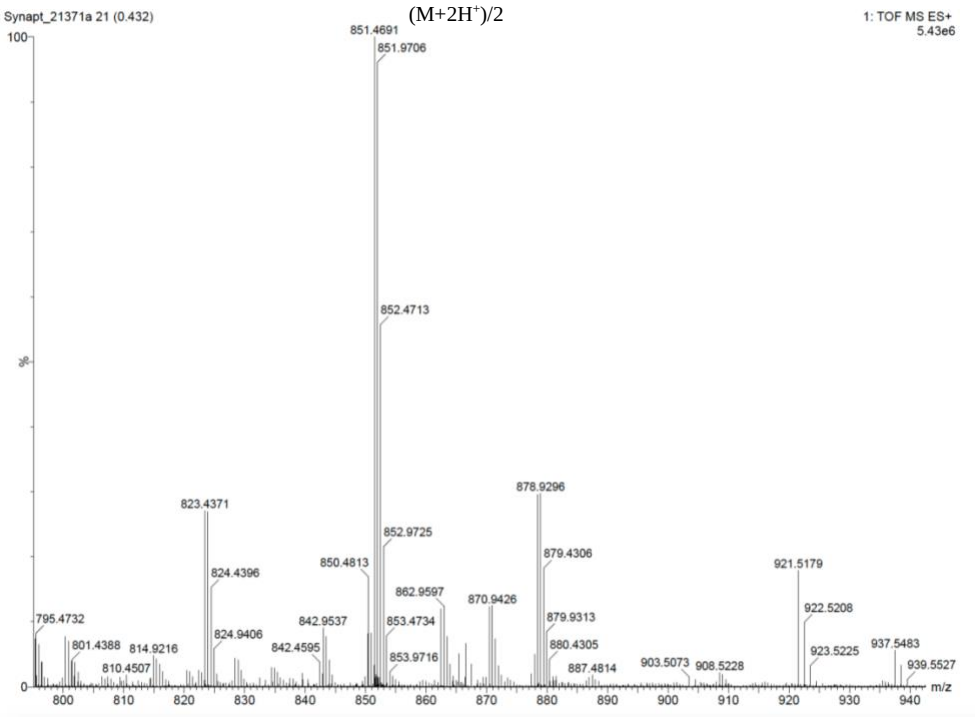
HRMS of Fmoc-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2



High resolution mass spectra of product (with protecting groups)

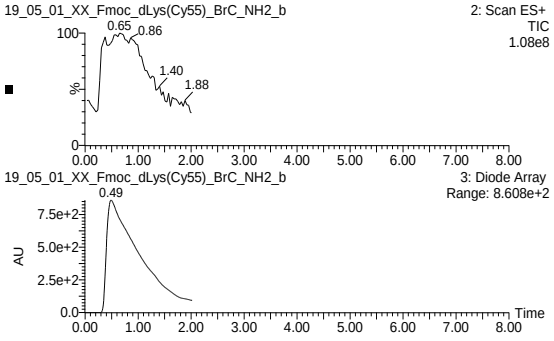


High resolution mass spectra of product (with protecting groups, 1701.9264 amu)

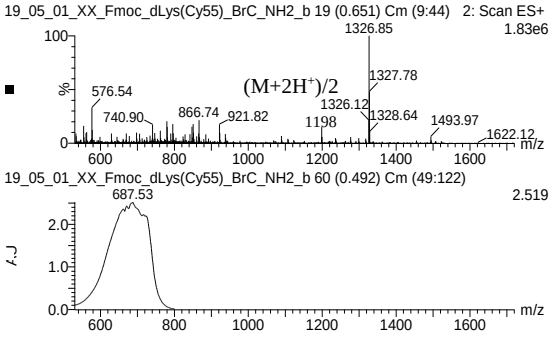


High resolution mass spectra of product (Half mass, with protecting groups, 851.4691 amu.)

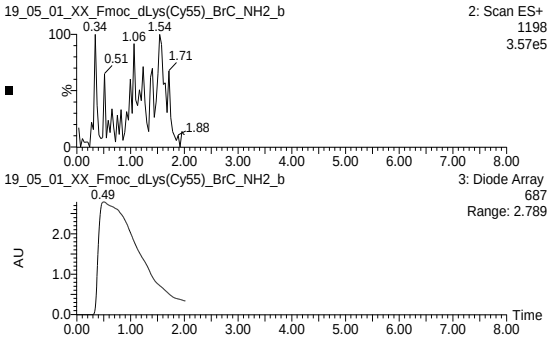
Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **23**



Total Ion Chromatogram (TIC)
LC-MS Total Diode Array Chromatogram (TDA)

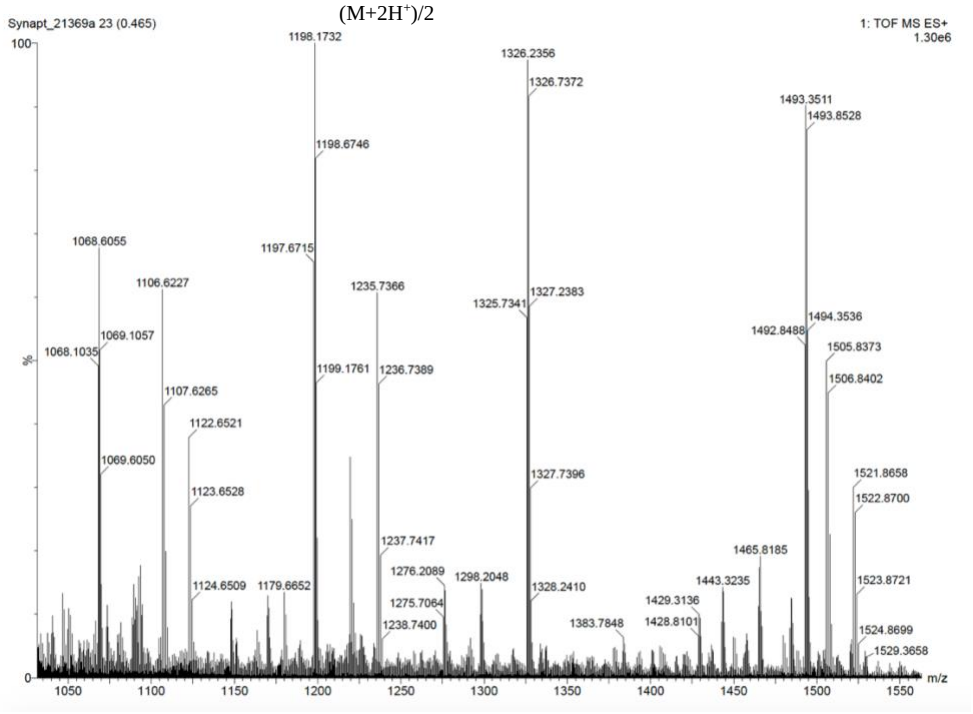


Mass Spectra and UV Spectra of cleaved product



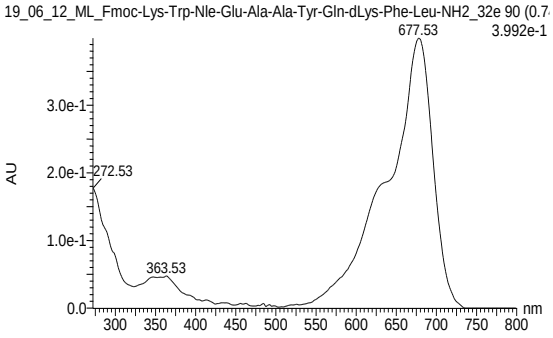
Extracted Ion Chromatogram (XIC) at 1326 amu.
Single Wavelength Chromatogram (SWC) at 687 nm

HRMS of Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2

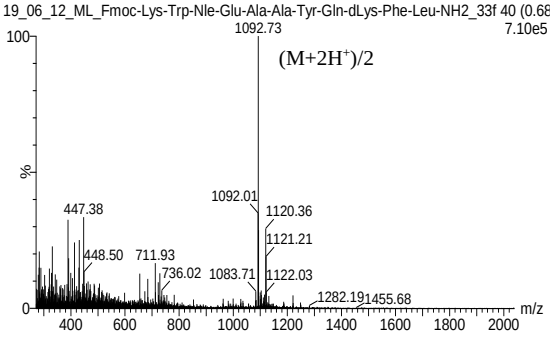


High resolution mass spectra of product (Half mass, with protecting groups, 1198.1732 amu.)

Fmoc-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2

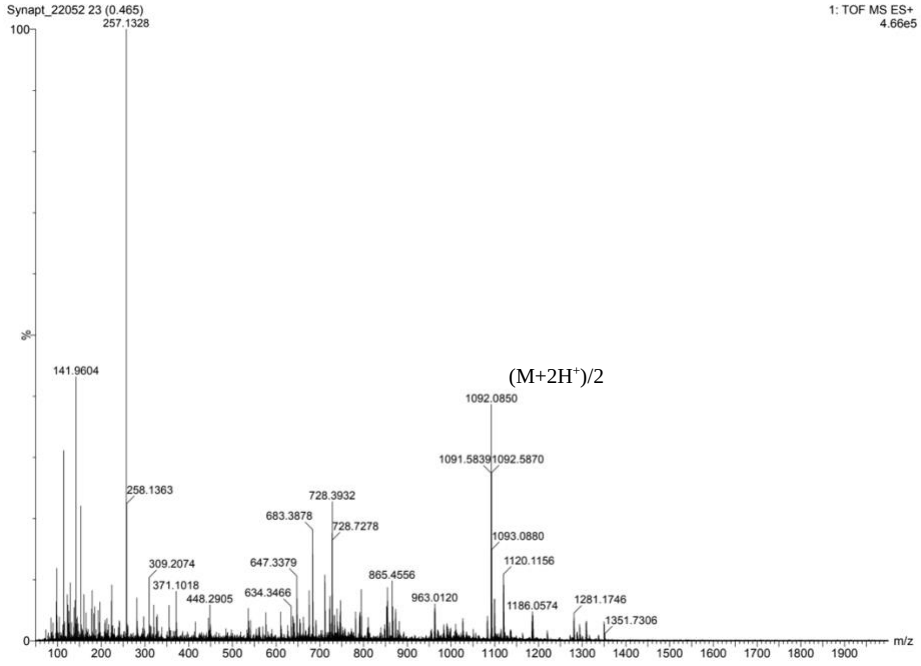


UV Spectra of cleaved product

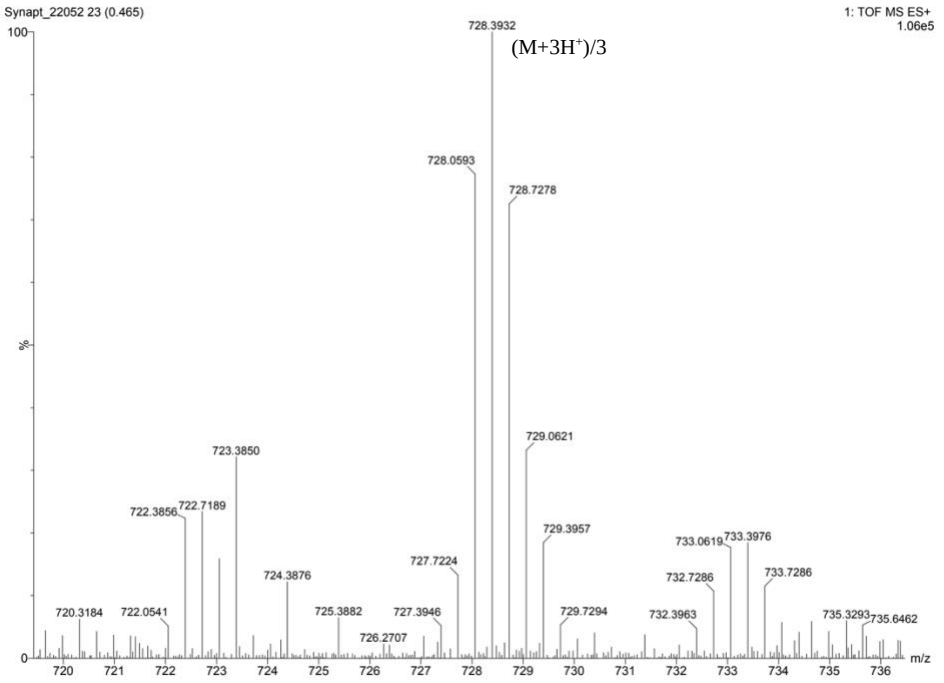


Mass Spectra of cleaved product (Half mass)

HRMS of Fmoc-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2

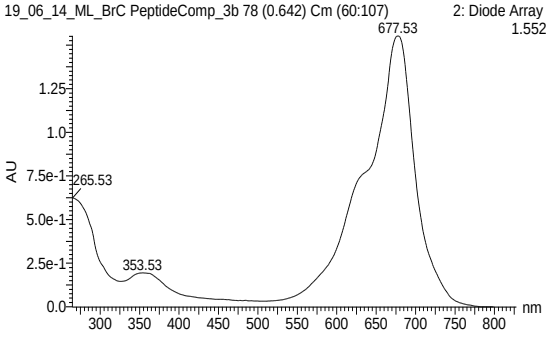


High resolution mass spectra of product (Half mass, without protecting groups, 1092.0850 amu.)

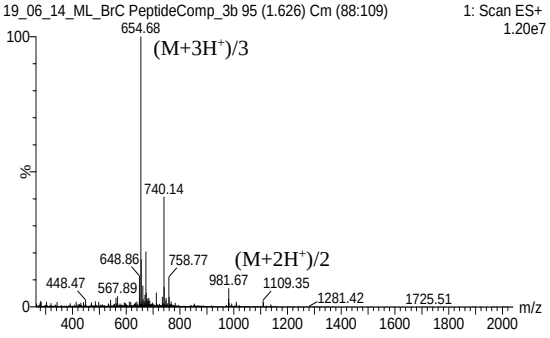


High resolution mass spectra of product (Third mass, without protecting groups, 728.3932 amu.)

H-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2 **24**

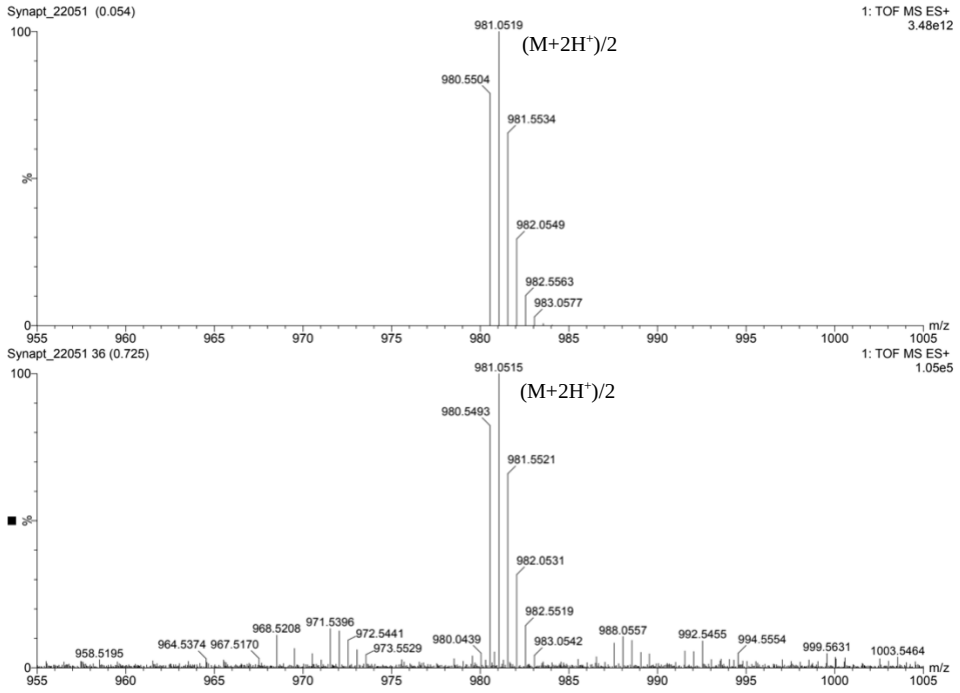


UV Spectra of cleaved product

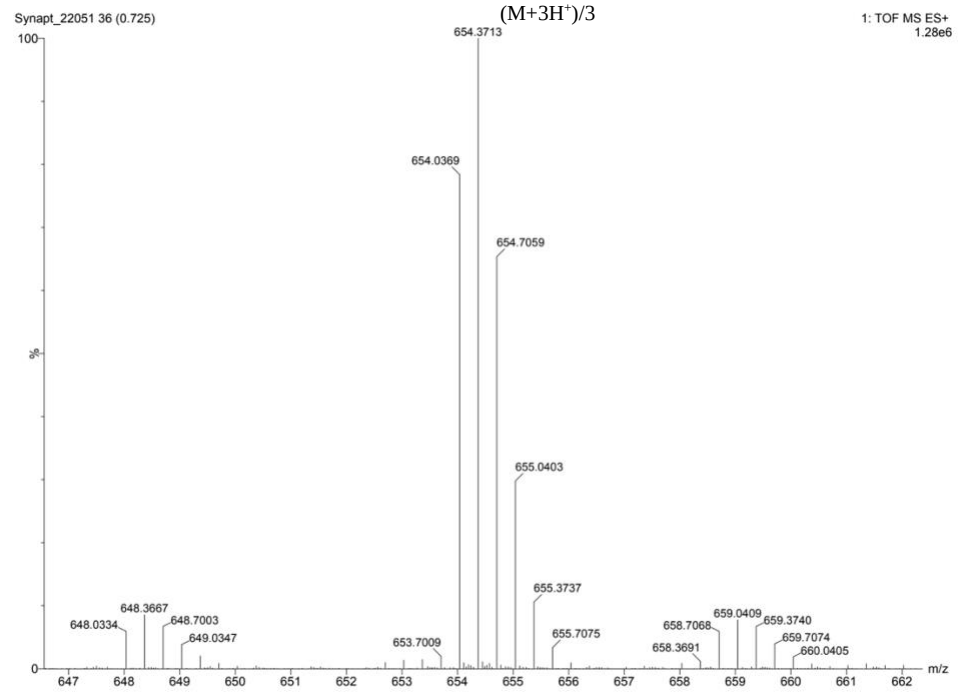


Mass Spectra of cleaved product (Half mass)

HRMS of H-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2

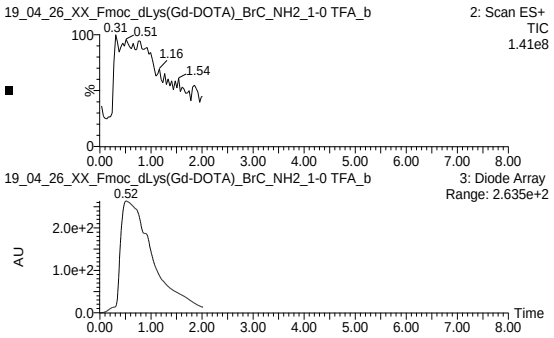


High resolution mass spectra of product (Half mass, without protecting groups, 981.0515 amu.)

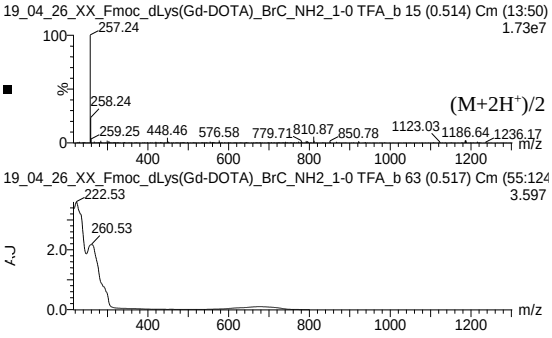


High resolution mass spectra of product (Third mass, without protecting groups, 654.3713 amu.)

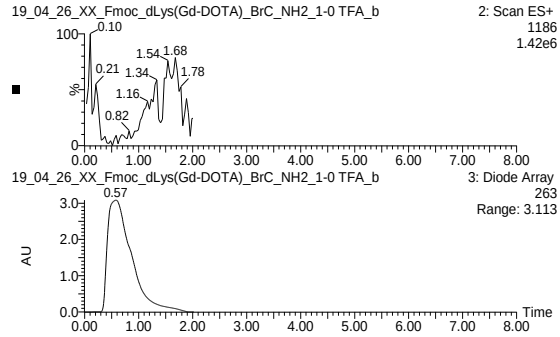
Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2 **25**



Total Ion Chromatogram (TIC)
LC-MS Total Diode Array Chromatogram (TDA)

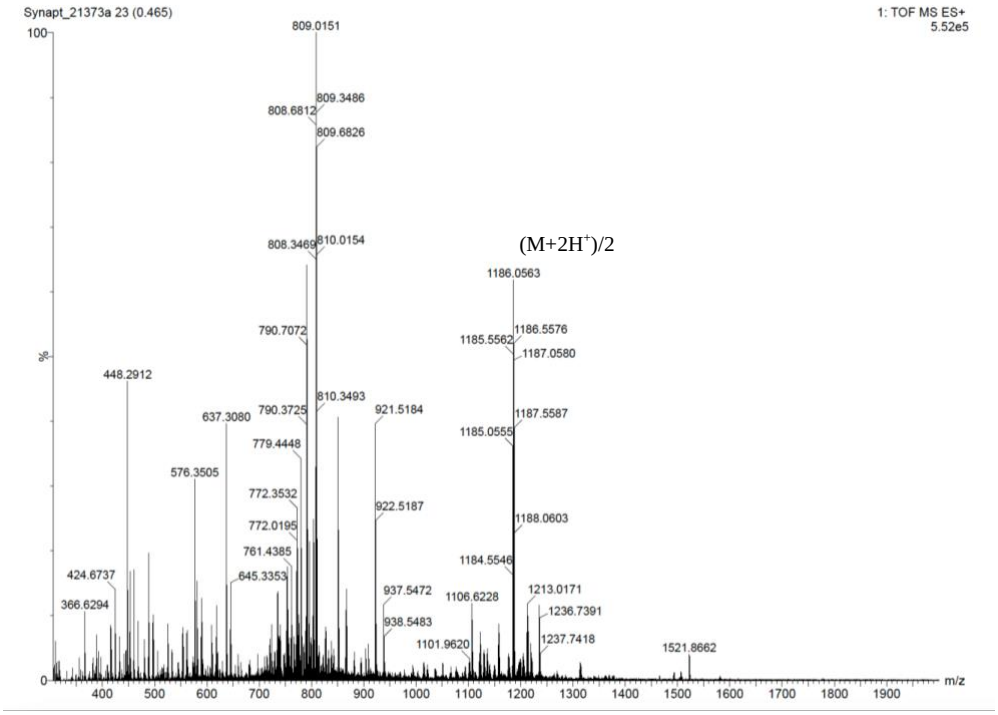


Mass Spectra and UV Spectra of cleaved product

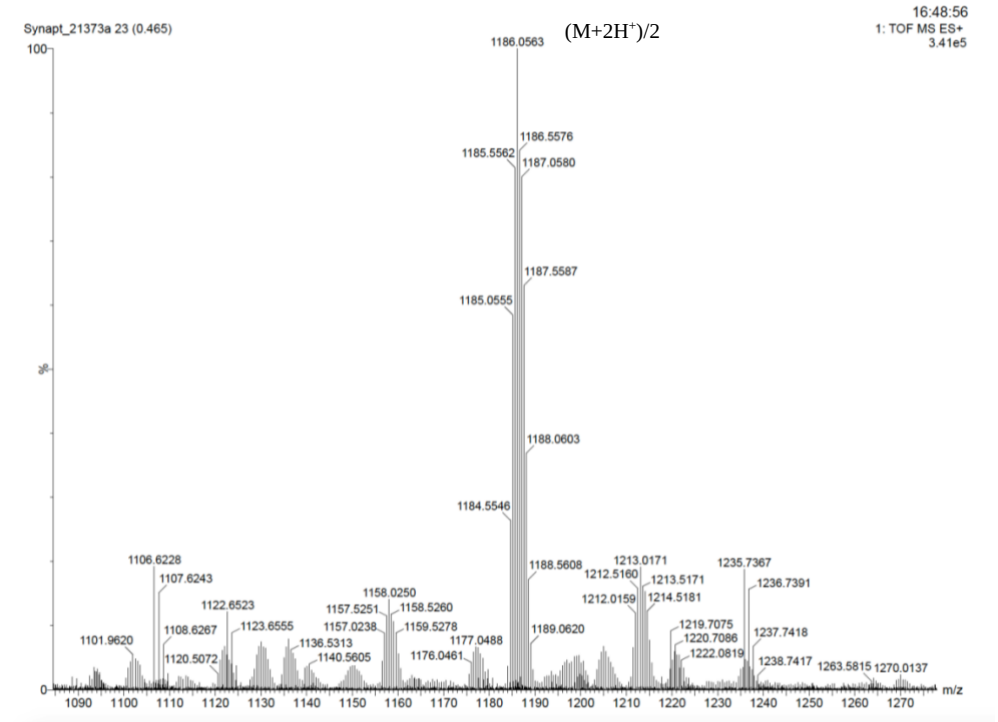


Extracted Ion Chromatogram (XIC) at 1186 amu.
Single Wavelength Chromatogram (SWC) at 263 nm

HRMS of Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2



High resolution mass spectra of product (Half mass, with protecting groups, 1186.0563 amu.)



Expanded high resolution mass spectra of product (Half mass, with protecting groups, 1186.0563 amu.)

Table 6: Molecular Weight of Peptides (Bold - Targeting peptides)

Compounds	Molecular Weight (g/mol)	Half Mass (g/mol)
Fmoc-Leu-NH_2	**352.2**	**177.1**
Fmoc-Phe-NH_2	386.2	194.1
Fmoc-Phe-Leu-NH_2	**499.2**	**250.6**
Fmoc-d-Lys(Mtt)-Phe-Leu-NH_2	883.5	442.75
Fmoc-d-Lys-Phe-Leu-NH_2	**627.3**	**314.65**
Fmoc-Gln-d-Lys-Phe-Leu-NH_2	**755.4**	**378.7**
Fmoc-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	974.5	488.25
Fmoc-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**918.5**	**460.25**
Fmoc-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1045.6	523.8
Fmoc-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**989.5**	**495.75**
Fmoc-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1116.6	559.3
Fmoc-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**1060.5**	**531.25**
Fmoc-Glu(OtBu)-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1245.6	623.8
Fmoc-Glu-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1245.6	623.8
Fmoc-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1301.7	651.85
Fmoc-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**1189.6**	**595.8**
Fmoc-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**1302.7**	**652.35**
Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1358.7	680.35
Fmoc-d-Nle-Glu-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1358.7	680.35
Fmoc-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1414.8	708.4
Fmoc-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**1488.7**	**745.35**
Fmoc-Trp-d-Nle-Glu(OtBu)-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	1544.8	773.4
Fmoc-Trp-d-Nle-Glu-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1544.8	773.4
Fmoc-Trp-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1600.9	801.45
Fmoc-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	1700.9218	851.46
Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	2394.3381	1198.17
Fmoc-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**2182.1610**	**1092.08**
NH_2-d-Lys(Cy5.5)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**1960.08800**	**981.05194**
Fmoc-d-Lys(Cy5.5)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	**2182.15990**	**1092.08589**
Fmoc-d-Lys(Gd-DOTA)-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-d-Lys-Phe-Leu-NH_2	**2167.9199**	**1084.96**
Fmoc-d-Lys(Gd-DOTA)-Trp(Boc)-d-Nle-Glu(OtBu)-Ala-Ala-Tyr(tBu)-Gln-d-Lys-Phe-Leu-NH_2	2370.0970	1186.05